

EXTRACELLULAR AMINO ACID EFFECTS ON MILK PROTEIN
SYNTHESIS AND FREE AMINO ACID POOLS IN CULTURED
RAT AND BOVINE MAMMARY CELLS

by

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INTRODUCTION

During the past six years, the average production of cows on test in the United States has increased 539 kg. The present record of 24,948 kg of milk in 365 days by Beecher Arlinda Ellen would indicate there are still significant gains in milk production to be achieved. Programs such as Virginia's Project 1700 that have the stated goal of accelerating increases in milk production are sure to reenforce the trend of higher individual animal production in the Unites States. To achieve these higher productions, dairy animals will have to be selected for more efficient utilization of nutrients. However, with present technology very little can be done to improve the action of the rumen microbes with respect to protein nutrition. The symbiotic relationship between dairy animal and rumen microbe, which is so beneficial when low quality feeds are fed, becomes a detriment with the high producing dairy cow fed high quality feeds.

Theoretical calculations and research results suggest cows producing over 30 kg of milk per day are experiencing amino acid insufficiencies for milk synthesis. The potential for a key nutrient to limit milk production will increase as higher productions are attained. The challenge to the nutritionist is to determine the nutrient requirements of the dairy animal and to provide for these requirements in the most economical way possible. The total requirement of the animal results from the summation of requirements for the various systems. In this dissertation the amino acid requirement of the mammary cell for milk protein synthesis in vitro is investigated.

This dissertation is an extension of the work conducted by Park (1975). In the first phase of the present research the ability of the mammary cell for the uptake of lysine and methionine and the effect of extracellular lysine and methionine on free intracellular amino acid pools was determined by using rat mammary cells in culture. Thereafter, nonspecific binding of labeled precursor amino acids to β -casein and β -lactoglobulin was considered as a possible error in measured milk protein synthesis. Several experiments followed using bovine mammary cells in culture. Syntheses of β -casein and β -lactoglobulin in response to graded quantities of amino acids in the medium was measured and the order of limiting amino acids in Eagle's minimal essential medium determined. The ability of the bovine mammary cell to concentrate culture medium amino acids was compared to the cell's requirement for those amino acids.

LITERATURE REVIEW

Amino Acid Insufficiencies for Lactation

When considering amino acid nutrition of dairy cows, McCarthy et al. (1970) makes the point that well-fed animals are not experiencing a true amino acid deficiency as they are producing milk and are in good health. Jacobson et al. (1970) estimates cows producing 30 kg of milk per day may be on the border of experiencing an amino acid insufficiency and higher producing animals might benefit from supplementation with specific amino acids. However, estimates by Chandler (1970) and Chandler and Polan (1970) on the availability of amino acids from natural feedstuff and rumen fermentation point to the possibility of different amino acids being in limited supply, depending on the feeding regime.

Results from research dealing with amino acid insufficiencies in lactating cows are quite variable and limited compared to work with other ruminants. Schwab et al. (1976) suggest amino acid insufficiencies for milk protein synthesis may not be as great as for wool growth and N retention in sheep since the amino acid profiles of rumen microbial protein and milk protein are very similar. The relatively high quality of rumen microbial protein for milk protein synthesis and the possibility of different amino acids being in limited supply would make the identification of limiting amino acids for milk protein synthesis extremely difficult. Research which has produced evidence for amino acid insufficiencies in lactating cows has resulted

from postruminal infusion of protein or amino acids.

Patterson (1972) observed that the nutrient requirements of a cell can be estimated fairly accurately from the composition of the product being formed. Casein, being the major fraction in milk, should contain an ideal amino acid complement for milk protein synthesis. Postruminal infusion of casein has resulted in significant increases in milk yield and milk protein production in work reported by Derrig et al. (1974), Vik-Mo et al. (1974), Clark et al. (1973), Spires et al. (1973), Hale et al. (1972), and Tyrrell et al. (1972). While milk protein production was consistently responsive to casein infusion, higher producing cows had the greatest increase in milk yield when casein was infused postruminally.

Broderick et al. (1970) infused 800 g of sodium caseinate plus 24 g of methionine per day into the abomasum. This treatment resulted in a 6.2% increase ($P < .10$) in milk protein content, and 11.6% increase ($P < .05$) in protein production, a 10% decrease ($P < .05$) in grain intake and a nonsignificant increase in milk production of 5.3%. The reduction in feed intake was probably due to the amount of sodium caseinate administered as other workers have not observed this depression. In this experiment and in Vik-Mo et al. (1974) dietary protein was fed in excess of NRC standards.

The results of these experiments show an improvement in milk production and milk protein synthesis in response to postruminal infusion of casein even with animals being fed protein in excess of their dietary requirement. Casein is probably not being used as an energy source as isocaloric infusion of glucose in some of these experiments

(Spires et al., 1973; Tyrrell et al., 1972; Clark et al., 1973) did not show the response that casein infusion showed. Response to casein administered postruminally may be due to supplying a single limiting amino acid or more likely supplying a group of amino acids which might be limiting milk production.

Experiments infusing essential amino acids either singly or in combination have not been consistently successful. Teichman et al. (1969) found no response in milk production, milk protein or milk fat production when L-methionine was infused in the jugular at 5, 10, or 20% of the expected milk methionine production. Fisher (1969) intravenously infused 13 g DL-methionine or 26 g DL-methionine plus 52 g L-Lysine per day and found no effect on feed intake, milk yield or milk protein production. Norman (1975) infused in the jugular vein methionine, methionine plus lysine and methionine plus lysine plus phenylalanine at 25% of the amino acid content of the pretreatment milk and observed no significant increase in milk yield. Later work by Fisher (1972) showed a significant ($P < .05$) increase in milk protein production when 11.2 g of DL-methionine was infused daily but no significant increase in milk yield. Infusing concentrations of methionine above 11.2 g in this experiment resulted in milk protein production below that observed with the 11.2 g infusion. Fisher (1972) also infused lysine and histidine. Infusing lysine had no effect on milk protein or milk yield, but histidine significantly ($P < .05$) reduced milk protein production.

Schwab et al. (1976) infused amino acids into the abomasum and observed some positive responses. Contrary to the finding of Fisher (1972), lysine infused into the abomasum significantly ($P < .05$) increased milk yield and milk protein. Milk protein response to lysine infusion was 16% of that observed with sodium caseinate infusion. Methionine infusion alone resulted in no response, but when infused with lysine accounted for 43% of the total response of milk protein observed with sodium caseinate. Increased milk protein production was observed when threonine or isoleucine was infused with lysine and methionine suggesting these amino acids were colimiting after lysine and methionine.

Factors such as dietary status of the animals and milk production contribute to the variability in response to amino acid supplementation observed in the work cited in this review. The fairly consistent improvement in milk production and milk protein synthesis in response to postruminal infusion of casein and the response observed by Schwab et al. (1976) with abomasal infusion of lysine and methionine point to the potential gains to be achieved with amino acid supplementation in the lactating dairy cow.

Amino Acids for Milk Protein Synthesis In Vitro

As Park (1975) recently reviewed in vitro synthesis of milk protein, this review will only consider the work which has measured milk protein synthesis in response to varying amino acids in the medium. Using rat and bovine mammary tissue, Schingoethe et al. (1967) identified the amino acids required by the mammary tissue of both species.

The usual list of amino acids for cultured mammalian cells (Eagle, 1957) was required except the rat tissue did not require glutamine or cystine and the bovine tissue did not require glutamine or tyrosine. Subsequent work showed phenylalanine hydroxylase activity in the bovine mammary tissue was sufficiently high to eliminate the tyrosine requirement in the medium (Jorgensen and Larson, 1968).

Besides a difference in essential amino acids required by the rat and cow mammary tissue, milk protein synthesized in response to graded concentrations of amino acids in the medium differed. Both rat and bovine mammary tissue showed a significant ($P < .05$) increase in β -casein synthesis with increased essential amino acids in the medium. However, β -lactoglobulin synthesis by bovine mammary tissue did not respond to increased essential amino acids. Though β -lactoglobulin has not been identified in rat's milk, a β -lactoglobulin like protein was analyzed and found to significantly ($P < .05$) increase with essential amino acids (Schingoethe et al., 1967). The lack of a significant response in β -lactoglobulin synthesis by bovine mammary cells might be due to the concentration synthesized on the control treatment (12.1 ug/flask). This value seemed higher than β -lactoglobulin reported by these researchers on similar experiments (Larson, 1969; Larson, 1972).

Park and Chandler (1976) culturing rat mammary tissue by the same procedure as Schingoethe et al. (1967) showed a linear response in β -lactoglobulin and β -casein to increased concentrations of medium amino acids up to 5 times their original concentration in Eagle's

minimal essential medium. The milk protein, α -lactalbumin, which was not measured by Schingoethe et al. (1967), increased with extracellular amino acids until a 3-fold concentration in the medium was reached.

Park et al. (1976b) conducted a series of experiments to determine the amino acid requirements of rat mammary cells for milk protein synthesis. One series of experiments was similar to that of Schingoethe et al. (1967). The full complement of amino acids in the medium was elevated 3-fold and in each treatment the concentration of one amino acid was decreased to its original concentration. One important difference in the two reports was that the results of Park et al. (1976b) would indicate that cystine is an essential amino acid for the rat mammary cell.

In a second series of experiments by Park et al. (1976b), each amino acid in the medium was elevated, individually, to 3 times its base level. Combining the results from both series of experiments, it was concluded that lysine was the first limiting amino acid in Eagle's minimal essential medium, methionine the second, followed by valine and arginine which seemed to be colimiting milk protein synthesis. Schingoethe et al. (1967) places threonine, tyrosine, histidine and lysine as the most likely to limit milk protein synthesis. The differences observed between these two reports and the differences observed between experiments in the studies by Park et al. (1976b) points to the large amount of variability in this type of work.

After identifying the limiting amino acids in Eagle's minimal essential medium for milk protein synthesis by rat mammary cells in culture, Park et al. (1976a) determined the optimum concentration of these amino acids. The results indicate lysine was required at 15 times its original concentration in the medium. This requirement for lysine was difficult to explain. The authors suggest that lysine might not be efficiently taken into the mammary cell, requiring large quantities in the medium in order to maintain an adequate intracellular concentration for protein synthesis. Another explanation pointed to the possibility of an error in measuring the effect of lysine on protein synthesis when the incorporation of labeled lysine is used to determine the amount of protein synthesized. These two explanations are further investigated in this dissertation. The potential for error in measuring β -lactoglobulin will be discussed later in this review.

The optimum response for methionine, valine and arginine was 4.5 times their original concentration in the medium. The methionine requirement agreed with Larson's (1972) work with bovine mammary cells in culture. The requirement for isoleucine, tryptophan, threonine, phenylalanine and histidine was 1.5 times their concentration in the original medium. Leucine, tyrosine, cystine, and glutamine concentrations in the original medium were adequate for milk protein synthesis.

Larson (1972) showed that elevating methionine in the medium with bovine mammary cells resulted in increased β -lactoglobulin and

β -casein syntheses. The fact that increasing the full complement of amino acids in a similar experiment by Schingoethe et al. (1967) did not significantly increase β -lactoglobulin seems contradictory and could not be explained by Larson (1972). The optimum methionone concentration in the medium seemed to be between 30 and 60 ug/ml (Larson, 1972). Caution must be taken when considering the results of this paper as no statistical analysis was run and mammary cells from different cows did not respond consistently to the methionine addition to the medium. Mammary tissue from 2 of the 6 cows used in this study showed no response to added methionine.

The work cited in this section demonstrates that the amino acid concentration in the medium affects the net synthesis of some components of milk protein. This would suggest that if sufficient amino acids could be made available to the mammary cell, it is possible to increase the milk protein yield in vitro. Postruminal supplementation of casein and individual amino acids cited in the previous section would support this view in vivo.

Analysis of Milk Protein Synthesis In Vitro

The two major milk proteins analyzed in this dissertation and the culture work just cited were β -casein, which accounts for 25 to 35% of the protein in bovine skim milk, and β -lactoglobulin accounting for 7 to 12% of bovine skim milk protein (Rose et al., 1970). The basic procedure used for measuring in vitro milk protein synthesis in this work was proposed by Groves and Larson (1965). By this procedure radioactive amino acids are incubated with the mammary cells

in culture. These amino acids are incorporated into the milk proteins being synthesized by the mammary cells. The radioactive milk protein is then coprecipitated with skim milk which is added to the culture flasks. The milk proteins then are separated by the salting out procedure of Aschaffenburg and Drewry (1957) and Aschaffenburg (1963). Knowing the amino acid composition of the milk proteins, the specific activity of the labeled amino acid and the amount of carrier protein added to the culture flask, the in vitro synthesis of milk protein can be calculated. One assumption being made is that the radioactivity associated with the milk protein is from labeled amino acids incorporated in the protein. Groves and Larson (1965) noted the radioactivities of proteins isolated from control groups which received isotope and then were immediately terminated without incubation were between .2 and 4.0% of the radioactivities of proteins from cultures which had been incubated for 18 hr.

Besides incorporation of amino acids into protein by biosynthesis, factors such as amino acid absorption, peptide bond formation, exchange or binding of labeled amino acid and protein decomposition will all affect the radioactivity associated with protein. Cornwell and Luck (1958) observed considerable uptake of lysine and phenylalanine by histones. By the use of dialysis 30 to 80% of the bound amino acid could be removed. However, this leaves a significant amount of amino acid more firmly attached. The binding they observed was a function of amino acid concentration, protein concentration, pH, temperature, and incubation time. Rotermund et al. (1970) tested the binding of

amino acids to rat serum albumin. Using L-(U-¹⁴C) lysine and L-(4,5³H) leucine, instantaneous labeling of albumin occurred. The bound activity could not be removed by dialysis indicating that the labeled amino acid was not simply absorbed onto the protein. The presence of ATP and its generating system increased the spontaneous binding of labeled amino acid to albumin. This work would suggest that under certain conditions nonspecific binding of labeled amino acids to milk proteins could introduce significant error into the calculated synthesis of the milk proteins.

Using Grove's and Larson's (1965) procedure, Schingoethe et al. (1967), Park and Chandler (1976), Park et al. (1976a), and Park et al. (1976b) reported measuring a β -lactoglobulin like protein in rat's milk, Lyster (1972) and Jenness (1970) question if β -lactoglobulin is being measured in these experiments as it has never been isolated from rat's milk. The potential for nonspecific binding of labeled amino acid in the medium with β -lactoglobulin in skim milk must be considered.

Bovine β -lactoglobulin is known to interact with organic ions. Lovrien and Anderson (1969) reported 4 binding sites for organic anions in β -lactoglobulin. They reported 2 very strong anion binding sites when β -lactoglobulin is under an acid condition and 2 binding sites on β -lactoglobulin when it is under alkaline conditions. Ray and Chatterjee (1967) reported on the binding of a dye anion (methyl orange), a long chain anion (dodecyl sulfate) and a long chain cation (dodecyl pyridinium) to β -lactoglobulin. Wishnia and Pinder (1966)

reported on a study involving the binding of butane, pentane and isobutane to β -lactoglobulin. The dissociation constants they reported indicate these alkanes are strongly bound. Based on these observations, the error in measured milk protein synthesis due to nonspecific binding of labeled amino acids deserves further investigation.

Intracellular Free Amino Acid Pools

Mammalian cells which are actively synthesizing protein have an increased concentration of free intracellular amino acids (Christensen et al., 1948a; Christensen et al., 1948b; Riggs et al., 1952; Riggs and Walker, 1958; Riggs and Walker, 1963; Mohri, 1967). Using ascites tumor cells, Riggs and Walker (1963) found a direct correlation between the concentration of free amino acid inside the cell and its incorporation into protein. From this work, Riggs and Walker (1963) concluded that incorporation of an amino acid into a protein depends on the available energy to the cell and the concentration of that amino acid free inside the cell.

The observation that intracellular amino acid pools increase with protein synthesis led to the assumption that these pools were the precursors for protein synthesis. However, this has been questioned. Kipnis et al. (1961) using a rat-diaphragm preparation looked at the kinetics of amino acid uptake and amino acid incorporation into cellular protein and concluded that the total free amino acid pools could not be the source of amino acids for protein synthesis. Kipnis et al. (1961) theorized that intracellular amino acid pools are compartmentalized. One possibility is that amino acids for protein synthesis are restricted to morphological sites such as the microsomes,

nuclei or mitochondria. Another possibility is that amino acids are chemically compartmentalized. Amino acids entering the cell destined for protein synthesis are immediately converted to acyl adenylates and esterified with soluble RNA.

Several authors (Riggs and Walker, 1958; Adamson and Anast, 1966; Hider et al., 1969; Samli et al., 1971) have observed that the rate of amino acid incorporation into protein is correlated with the rate of the amino acid transport, which would not support the concept of the free amino acid pools serving as the precursors to protein synthesis. This has led to the theory that the selection of amino acids for protein synthesis occurs while they are associated with the transport system of the cell.

Adamson et al. (1972) expressed this theory of amino acid selection in the following way. Amino acids are selected for protein synthesis while bound to the carrier protein in the cell membrane. When the concentration of amino acids becomes great enough the incorporation of amino acids into protein becomes saturated and the membrane amino acid complex dissociates with the release of amino acids inside the cell. The amino acids released intracellularly can reassociate with the transport system but must compete with extracellular amino acids for transport sites. This theory allows both intracellular and extracellular amino acids to serve as precursors of protein synthesis. The potential for amino acid transport to limit protein synthesis would seem high for amino acids which have very small intracellular pools, such as tryptophan or methionine (Munro, 1970) or amino acids

which are not efficiently concentrated in the cell, such as arginine or lysine (Eagle and Piez, 1962).

The concentration of amino acid pools in the cell depends in part on the extracellular amino acid concentrations (Eagle and Piez, 1962; Kuchler, 1964). The lower the extracellular concentration of an amino acid the more efficiently it is concentrated in the cell (Eagle and Piez, 1962). Most essential amino acids are concentrated 2- to 10-fold inside the cell when external concentrations are at physiological normals (Piez and Eagle, 1958). Nonessential amino acids are concentrated to a much greater extent than essential amino acids (Kuchler, 1964). Threonine seemed to act more like a nonessential amino acid in its uptake, as it was very actively concentrated by the cell (Kuchler, 1964; Eagle and Piez, 1961). The efficiency with which an amino acid's intracellular pool is maintained will partially determine the cell's requirement for that amino acid.

Amino Acid Transport

The potential for controlling protein synthesis through amino acid transport has been mentioned. Amino acid transport systems in the mammary cell have not been identified. Much of what is known about amino acid transport in mammalian cells results from the work of Christensen at the University of Michigan. Most of his work was done with Ehrlich ascites tumor cells. However, the basic concepts developed with these cells can probably be applied to all mammalian cells.

Several symposium papers on amino acid transport have been written by Christensen and are discussed here (Christensen, 1965; Christensen,

1969; Christensen, 1973). Amino acids are carried by different transport systems in Ehrlich ascites tumor cells. The specificity of the systems vary. The glycine system is specific for transport of glycine and sarcosine, while the A system transports all of the neutral amino acids (Christensen, 1969). Most amino acids are carried by more than one transport system, with several amino acids being transported by three separate systems (Christensen, 1973). The affinity of an amino acid for a system will vary. The external concentration of an amino acid will partially determine the extent it is carried by different systems. Amino acids transported by the same system will inhibit each other's transport by that system (Christensen et al., 1952). This was further demonstrated in the work by Oxender and Christensen (1963) measuring the inhibitory effect of several amino acids on glycine, alanine, leucine, and arginine. More recently the transport system for basic amino acids was found to be inhibited by neutral amino acids, in the presence of high concentrations of sodium ions (Thomas et al., 1971). The sodium ion concentration was greater than physiological concentration so this is a special case which would probably not occur in vivo.

In addition to competitive inhibition, competitive stimulation of amino acid transport is known to occur. Jacquez (1963) has shown several neutral amino acids to stimulate the uptake of tryptophan by Ehrlich cells. It was noted that as the length of the carbon chain increased so did the stimulatory effect. Schafer and Jacquez (1967) using Ehrlich cells found methionine to stimulate the transport of

tryptophan and phenylalanine. Christensen et al. (1952) showed arginine or lysine to stimulate the uptake of glycine, alanine, histidine and phenylalanine. The mechanism for this stimulation is not known. However, it appears that the maximum stimulation occurs when the stimulating amino acid and the stimulated amino acid are approximately equimolar. From this review it is evident that, altering the extracellular concentration of one amino acid not only affects its availability to the cell but the availability of other amino acids as well.

Ribonucleic Acid - Free Amino Acid Pool Interaction

Neonatal development of organs follows a developmental sequence. Cell DNA increases followed by RNA, then cellular protein synthesis increases. Just prior to the increased protein synthesis, free amino acid pool size increases. Miller (1970) speculates that the role of the gene is to serve as a course control for protein synthesis, while available amino acids determine the exact rate of protein synthesis reached.

Mohrenweiser and Emery (1973) using rat mammary tissue showed they could increase RNA content in the cell by increased amino acid concentration in the medium. The increased RNA observed in this experiment was probably due to decreased catabolism of RNA and not to increased synthesis, as assumed by the authors. Enwonwu and Munro (1970) have shown that RNA catabolism is decreased by increasing available amino acids to mammalian cells in culture.

The role of amino acids in regulating ribosome function has been investigated by Munro (1968). Using the liver cell of the rat in vivo,

he found that polysome aggregation or disaggregation seemed to be closely linked to availability of amino acids. Tryptophan seemed to be the key amino acid in the whole animal, but subsequent work showed other amino acids could change the polysome profile and regulate RNA catabolism (Munro, 1970). As polysome size is positively correlated with the incorporation of amino acids into protein, it is interesting to speculate that free amino acids are not only serving as precursors of protein synthesis but also as regulators of protein metabolism.

Areas Requiring Further Research

The work cited in this review indicates that under certain conditions lactating dairy cows can increase milk protein synthesis in response to increased amino acids. However, in the literature reviewed there was little agreement concerning the identification of limiting amino acids for milk protein synthesis. Considering the large number of variables contributing to amino acid metabolism in the ruminant in vitro studies with mammary cells would be an expedient way to identify the mammary glands response to supplementation of specific amino acids.

The synthesis of β -lactoglobulin by bovine mammary cells in culture was increased by the addition of methionine but not by the addition of the full complement of amino acids in Eagle's minimal essential medium (Larson, 1972; Schingoethe et al., 1967). These responses in β -lactoglobulin seem contradictory and merit further investigation. If milk protein fractions respond differently to amino acid supplementation the biological value of milk protein would change and this

would be important when considering amino acid supplementation of the whole animal.

The amino acids required by bovine mammary cells in culture have been identified (Schingoethe et al., 1967). Measuring bovine milk protein synthesis in culture and changes in intracellular amino acid pool size in response to increased concentrations of required amino acids would give an indication of the required concentration for these amino acids. The results of such an experiment would serve as a reference point for further in vivo studies. It would seem from this review that several basic questions concerning amino acid supplementation of lactating dairy cows can most efficiently be answered with in vitro techniques.

MATERIALS AND METHODS

Response of Free Amino Acid Pools in Rat Mammary Cells to Extracellular Lysine and Methionine

Mammary tissue from Sprague-Dawley rats, 2-5 days postpartum, was used. Euthanasia of rats was with ether. Mammary tissue was removed and placed in sterile balanced salt solution with antibiotics (penicillin-G 100 U/ml, streptomycin sulfate 100 µg/ml, and nystatin 60 µg/ml). Tissue was finely minced and digested with collagenase prior to culturing by the procedure developed by Ebner et al. (1961). The basic culture medium was Eagle's minimal essential medium (MEM) with 20% increase in glucose and 7% bovine serum (Table 1). A complete description of the culture procedure, preparation of medium and other solutions used is given by Park (1975).

Four replications of a randomized block were used with treatments shown in Table 2. Viable nuclei were stained for counting with crystal violet in citric acid and approximately 1×10^7 cells were added to each culture flask (Bryant et al., 1958). The uptake of methionine and lysine was measured by adding to the medium in each flask .6 uCi of either L-[U-¹⁴C] methionine or lysine. After culturing 18 hr, the contents of five flasks, containing the same treatment and labeled amino acid, were pooled into a 50 ml centrifuge tube.

Cell-medium mixture then was centrifuged at 200 x g for 3 min and the medium removed from cells by pipetting. Cells were washed with 10 ml of balanced salt solution. Five ml of cold (~10 C) 8% trichloroacetic acid (TCA) were added to the centrifuge tube to precipitate protein and release intracellular amino acids. The contents

Table 1. Composition of Eagle's minimal essential medium^a

Components		Components	
L-Amino Acids	(mg/L)	Glucose ^b (mg/L)	1,200.0
Arginine	105.0	Inorganic Salts ^c	
Histidine HCl-H ₂ O	41.9	Vitamins ^d	
Isoleucine	52.5	Supplements	
Leucine	52.4	Bovine Serum (ml/L) ^e	70.0
Lysine HCl	73.1	Antibiotics ^f	
Phenylalanine	33.0	¹⁴ C-(U)-amino acids ^g	
Threonine	47.6		
Tryptophan	10.2		
Valine	46.8		
Methionine	14.9		
Tyrosine	36.2		
Cystine	24.0		
Glutamine	292.0		

^aMinimum Essential Medium (Eagle) Cat. No. 12-901. Microbiological Associates, Inc., Bethesda, Maryland.

^bConcentration shown was the adjusted value by 20% increase from what was given in MEM (Eagle, 1959).

^cEarle's Balanced Salt Solution.

^dThe same components as defined in the MEM.

^eThe added 7% (V/V) serum to the MEM.

^fPenicillin-G, streptomycin sulfate, and nystatin at levels of 100 u/ml, 100 µg/ml, and 50 u/ml.

^gL-Lysine, leucine or methionine incorporated at ≈60 µCi/L.

Table 2. Treatments for effect of lysine and methionine on free amino acid pools

Treatment	Composition of media
1X	Eagle's Minimal Essential Medium (MEM)
1X + Methionine	MEM + 4X Methionine (74.5 mg/l)
1X + Lysine	MEM + 4X Lysine (233.9 mg/l)
5X	MEM + 4X all MEM amino acids

pH adjusted to 7.4 with 7.5% solution of sodium bicarbonate.

of the centrifuge tubes were mixed and centrifuged at 10,000 x g for 10 min. The amino acids in the supernatant were assumed to represent the intracellular amino acids and were separated after centrifugation from the insoluble material by pipetting (Eagle et al., 1961). Supernatant then was extracted with diethyl ether to remove TCA and soluble lipids. One ml of the TCA extract, containing the free amino acids, was counted to determine intracellular radioactivity from L-[U-¹⁴C] lysine and methionine. The remainder of the intracellular amino acid solution was lyophilized, followed by reconstitution in pH 2.0 lithium buffer. The amino acids in this sample were analyzed by automated ion exchange chromatography and the results used to calculate the free intracellular amino acid pools. An additional aliquot was subjected to paper chromatography (Hawk et al., 1951) and liquid scintillation counting to determine the specific activity of intracellular lysine and methionine.

In two of the replications, the distribution of label between media and cell protein was determined. A .1 ml aliquot of medium taken before and after incubation, was counted. The TCA precipitate from incubated cells was extracted with 10 ml of hot (~50 C) TCA followed by extraction with 10 ml of ether. This residue, which was assumed to contain the cell protein, was counted by liquid scintillation.

Analyses of variance were run on the labeled amino acid uptake data to determine the significance of treatment effect. Four observations were present for each treatment with each observation

representing the combined analysis of five flasks. Treatments were further compared by orthogonal contrasts with significance taken at the .05 level.

Nonspecific Binding of Labeled Lysine and Leucine to Milk Protein

To determine the extent of binding by L-[U-¹⁴C] lysine and leucine to β -lactoglobulin and β -casein, 1 ml of balanced salt solution, in place of cell suspension, was added to 5 ml of medium containing a 1-, 3-, 5-, or 7-fold complement of amino acids found in MEM. Then either .6 uCi of labeled lysine or leucine was added to the culture flask and the mixture incubated at 37 C for 18 hr. After incubation, 5 ml of skim milk was added to each culture flask and the contents of 3 flasks pooled. Labeled β -lactoglobulin and β -casein then were isolated with their skim milk fraction by the salting out procedure of Aschaffenburg and Drewry (1957) and Aschaffenburg (1963). A complete description of the isolation procedure is given by Park (1975). Duplicate measurements for both labeled amino acids in each of the amino acid complements were made. Analysis of variance was run with significance taken at the .05 level.

Interaction Between Extracellular Lysine and Leucine with Labeled Lysine and Leucine in the Medium

Mammary tissue used in this and subsequent studies was from Holstein cows belonging to Virginia Polytechnic Institute and State University herd which were being culled for reasons judged nondetrimental to the mammary tissue. Three Holstein cows (ave. 11.6 kg milk/day) were stunned by a mechanical stunner and were immediately ensanguinated. The mammary gland then was excised and pieces of interior secretory

tissue removed aseptically from each quarter. Secretory tissue was placed in balanced salt solution with antibiotics and packed in ice. Approximately 30 min elapsed from the time of slaughter until the tissue arrived at the laboratory. The culturing procedure and preparation of solutions was the same as in the previous rat study. Two replications of a 2 x 3 factorialized randomized complete block design was run. Mammary tissue from each cow served as a block. Treatments are shown in Table 3. Each measurement of β -lactoglobulin and β -casein represents the composite analysis of the contents of 3 flasks. Approximately 1×10^7 cells were added to each culture flask prior to the 18 hr incubation period. Analysis of variance was run to determine the significance of main effects and interaction. Treatments were further compared by orthogonal contrasts with significance taken at the .05 level.

Extracellular Amino Acid Effects on In Vitro Bovine Milk Protein Synthesis and Intracellular Amino Acid Pools

Mammary tissue from two Holstein cows (ave. 22.3 kg milk/day) was used. Tissue was handled the same as in the previous study and cultured by the procedure of Ebner et al. (1961). A randomized complete block design was run with mammary tissue from each cow representing a block. The basic culture medium was MEM with 20 percent increase in glucose and 7 percent serum (Table 1). The 4 treatments contained 1-, 3-, 5-, and 7-fold concentration of MEM amino acids (Table 4). The pH of the medium was maintained between 7.2 and 7.7 by the addition of sodium bicarbonate (7.5 percent). When sodium bicarbonate was added

Table 3. Treatments for effect of labeled amino acid on β -lactoglobulin and β -casein measurement

Treatment	Amino Acid Complement	L-[U- ¹⁴ C] Amino Acids ^a
1	MEM ^b	Lys
2	MEM	Leu
3	MEM + Lys ^c	Lys
4	MEM + Lys	Leu
5	MEM + Leu ^d	Lys
6	MEM + Leu	Leu

^aLabeled amino acid added to each flask was .6 μ Ci.

^bEagle's minimal essential medium (MEM) with 20% increase in glucose and 7% dialyzed bovine serum.

^cMEM plus .730 mg/flask of L-lysine HCl.

^dMEM plus .524 mg/flask of L-leucine.

Table 4. Treatments for extracellular amino acid effects on milk protein and intracellular amino acids

Treatment ^a	Block ^b	Complement of Amino Acids	pH ^c	mOsm/kg
1	1	1-fold MEM	7.3	321
1	2	1-fold MEM	7.7	299
2	1	3-fold MEM	7.6	249
2	2	3-fold MEM	7.3	327
3	1	5-fold MEM	7.6	350
3	2	5-fold MEM	7.4	340
4	1	7-fold MEM	7.2	380
4	2	7-fold MEM	7.3	354

^aBasic medium used in all treatments was Eagle's MEM.

^bMammary tissue used in block 1 came from a cow producing 21.8 kg milk/day. Mammary tissue used in block 2 came from a cow producing 22.7 kg milk/day.

^cSodium bicarbonate (7.5%) was added to medium to adjust pH.

to the medium a corresponding reduction in the other salts in the medium was required to maintain osmolarity. The viability of cultured cells was determined by staining with citric acid-crystal violet and counting cells before and after incubation (Bryant et al., 1958).

Contents of 3 culture flasks and 15 ml of skim milk were pooled for a single measurement of β -casein and β -lactoglobulin synthesis. Either L-[U- 14 C] lysine or leucine (.6 uCi/flask) was added to the medium with its subsequent incorporation into milk protein used to calculate net synthesis. Isolation of β -casein and β -lactoglobulin has been discussed (Park, 1975). Control flasks which contained 1 ml of salt solution rather than 1 ml of cell suspension were taken through the procedure. Radioactivity associated with the milk protein from control flasks was subtracted from radioactivity measured with cell cultures. In each block, measurement of β -casein and β -lactoglobulin for each treatment was in triplicate.

The contents of 5 culture flasks containing the same treatment were pooled to determine intracellular amino acids. The procedure for freeing intracellular amino acids and their measurement was the same as described earlier for the rat study. Prior to freeze drying, 1 ml of the TCA extract and an aliquot of the TCA precipitate were analyzed for nitrogen. Duplicate measurements of pools on each treatment were made. Significance of treatment effects on milk protein synthesis and intracellular pools was determined by least squares analysis. Regression equations for individual amino acid pools also were determined by least squares analysis.

Limiting Amino Acids for Milk Protein Synthesis by Bovine Mammary Cells
in Culture

Mammary tissue from 10 Holstein cows producing at least 11.4 kg of milk daily with an average of 12.7 kg was used. Handling of tissue and culturing were as in previous studies. A randomized complete block design with 10 blocks and 14 treatments was used to identify the limiting amino acid in MEM for milk protein synthesis. Mammary tissue from one Holstein cow was used in each block with approximately 8×10^6 cells added to each culture flask. Treatments consisted of a control (MEM) and in subsequent treatments a single amino acid elevation (3-fold) for each of 13 amino acids in MEM (Table 1). Uniformly labeled L- ^{14}C - leucine was the tracer amino acid used in 5 blocks and L-[U- ^{14}C] lysine was used in the other 5 blocks. The effect of L-lysine addition to the medium was not measured in the blocks using L-[U- ^{14}C] lysine nor was the effect of L-leucine addition measured in the blocks using L-[U- ^{14}C] leucine. Significance of treatment effects was determined by the method of least squares analysis. To determine the combined effect of treatments of β -casein and β -lactoglobulin synthesis, linear combinations of β -casein and β -lactoglobulin means were constructed for each treatment as described by Kramer (1977). Treatment effects then were compared using Duncan's new multiple-range test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Response of Free Amino Acid Pools in Rat Mammary Cells to Extracellular Lysine and Methionine

Table 5 compares the relative uptake of labeled lysine and methionine by mammary cells cultured in different treatments. Increasing concentrations of lysine or methionine in the medium resulted in a significantly higher intracellular radioactivity for that amino acid. Intracellular lysine and methionine were dependent on their exogenous concentration, indicating there was no restriction in the transport of these two amino acids by the mammary cell at the levels studied. Since lysine was effectively taken up by the mammary cell, limitations in the transport would not explain the high lysine requirement previously reported (Park et al., 1976a).

Comparisons between treatments containing the 1X concentration of methionine (1X vs. 1X + Lysine) or lysine (1X vs. 1X + Methionine) were not significant. However, comparisons between the treatments containing the 5X concentration of methionine (5X vs. 1X + Methionine) or lysine (5X vs. 1X + Lysine) were significant ($P < .05$). Intracellular radioactivity was highest when lysine or methionine were increased individually in the medium. This might suggest more efficient utilization of lysine and methionine when the concentration of these two amino acids increased with the full complement of amino acids. However, addition of lysine or methionine to the medium stimulated milk protein synthesis almost as well as when the full complement of amino acids was increased, indicating these two amino

Table 5. Uptake of ^{14}C labeled lysine and methionine by mammary cells^a

<u>Treatment</u>	Lysine- ^{14}C U		Methionine- ^{14}C U	
	<u>Mean</u>	<u>SE</u>	<u>Mean</u>	<u>SE</u>
1X ^b	1.00 A ^c	.08	1.00 A	.02
1X + Methionine	.85 A	.09	6.47 aB	1.41
1X + Lysine	5.61 aB	.23	.99 A	.17
5X	2.88 ab	.42	4.26 ab	.47
Radio-purity ^d	97.8%	1.3	92.4%	2.8

^aData expressed as a multiple of the 1X treatment. Disintegrations per minute (DPM) were corrected for unequal specific activity.

^bMean DPM for 1X, labeled lysine was 2712, and 1160 for 1X labeled methionine.

^cOrthogonal comparisons; within column mean with upper case letter significantly ($P < .05$) different from mean with lower case letter.

^dPaper chromatographic analysis (Hawk et al., 1951).

acids were utilized efficiently when increased individually (Park et al., 1976b). Also there is the possibility that competitive uptake of amino acids occurred resulting in decreased uptake of methionine and lysine by cells in the 5X treatment.

Based on the results of the first two replications, 99.95 ± 1.29 percent of the radioactivity from labeled lysine and 99.79 ± 1.45 percent of the radioactivity from labeled methionine was still in the medium after the 18 hr incubation period. Experiments which measure the disappearance of a nutrient from the medium generally have an incubation period longer than the incubation period in this study and the cells are rapidly growing and dividing with a rate of protein synthesis much greater than that of mammary cells in culture.

The TCA precipitate accounted for $1.23 \pm .27$ percent of the total radioactivity when labeled lysine was added to the medium and $.67 \pm .26$ percent when labeled methionine was used. The radioactivity associated with the TCA precipitate was probably more indicative of nonspecific binding of labeled amino acid to cell walls and cell debris in the medium than to actual protein synthesis. Intracellular radioactivity accounted for $.62 \pm .16$ percent of the radioactivity when lysine was the labeled amino acid in the medium and $.64 \pm .03$ percent when methionine was the labeled amino acid used.

The approximate ratio of medium to cell volume was 500 to 1 (Eagle and Levinton, 1965). Considering the external concentration of amino acids to the relatively small volume inside the cells, the

rate of protein synthesis and the short incubation time, the lack of a significant reduction in medium radioactivity was not surprising. As the variation due to counting masks any distribution shifts in labeled amino acid between treatments, the distribution of label was determined only on the first two replications.

The metabolism of amino acids begins soon after they enter the cell and will alter the intracellular pool size. On all four treatments containing labeled lysine, 92.3 ± 1.8 percent of the intracellular radioactivity was associated with the lysine intracellular pool following incubation. Only 42.2 ± 10.3 percent of the intracellular radioactivity was associated with intracellular methionine when labeled methionine was added to the medium. The percentage of radioactivity associated with intracellular methionine did not differ significantly between treatments indicating metabolism was at a rate dependent on methionine concentration in the medium.

Total intracellular amino acid pools as measured by ion exchange chromatography are given in Tables 6 and 7. The amino acids in Table 7 are not present in MEM and must be synthesized endogenously by the mammary cell or associated with the in vivo cell. However, there is probably free exchange of these amino acids between cells as they will leak from the mammary cell and be reconcentrated inside the cell. These amino acids will probably be more efficiently concentrated by the cell than the amino acids in MEM because of their low concentration outside the cell (Kuchler, 1964). Nevertheless, the contribution of endogenously synthesized amino acids to the total intracellular amino acids varied from 56.8% on the 1X MEM treatment to 26.6% on the 5X MEM treatment. This change was

Table 6. Mole percent of MEM intracellular amino acids in rat mammary cells^a

Amino Acid	Treatment			
	1X	1X + Lysine	1X + Methionine	5X
Arg	.56 ± .55	3.57 ± 1.19	.48 ± .47	10.53 ± 1.74
1/2Cys ^b	.51 ± .07	.37 ± .16	.87 ± .15	.93 ± .41
Met	-	-	-	-
Ile	7.91 ± .46	7.54 ± .96	9.74 ± 1.23	15.30 ± .59
Leu	5.79 ± .48	5.74 ± .58	7.16 ± .35	8.98 ± .35
Phe	3.08 ± .27	2.39 ± .48	3.71 ± .45	4.78 ± .17
Thr	4.82 ± .78	4.84 ± .76	7.60 ± 1.04	6.69 ± 1.26
Trp ^c	-	-	-	-
Tyr	3.81 ± .54	3.21 ± .37	2.95 ± .82	3.82 ± .73
Val	5.01 ± .21	4.67 ± .56	5.96 ± .62	8.38 ± .59
Lys	8.68 ± 1.35	21.42 ± 2.42	6.15 ± 1.52	9.73 ± .54
His	3.06 ± .40	2.50 ± .25	2.98 ± .29	4.08 ± .17

^aMean of four observations ± SE.

^bBelow detection limits of analyzer (less than 2 nanomoles).

^cBelow detection limits of analyzer (less than 5 nanomoles).

Table 7. Mole percent of intracellular amino acids not present in MEM in rat mammary cells^a

Amino Acid	Treatment			
	1X	1X + Lysine	1X + Methionine	5X
Asp	2.32 ± .39	1.87 ± .53	2.40 ± .29	1.19 ± .39
Ser	2.86 ± .46	4.10 ± 1.21	4.08 ± .61	3.67 ± 1.29
Glu	28.68 ± 8.80	19.35 ± 8.13	26.10 ± 8.36	11.09 ± 4.19
Gly	7.47 ± 1.30	6.00 ± .92	6.20 ± .95	3.39 ± 1.19
Ala	7.10 ± 1.00	6.58 ± .96	6.82 ± 1.07	1.96 ± .41
Orn	8.37 ± 1.90	5.86 ± 1.71	6.84 ± 1.63	5.25 ± 2.28

^aMean of four observations ± SE.

due primarily to an increase in the concentration of amino acids found in MEM and not to a decrease in synthesis of non-MEM amino acids by the cell.

Tryptophan and methionine had no measurable intracellular pools (Table 6). The reason for the lack of a tryptophan pool was probably due to its low concentration in the medium which results in a low quantity inside the cell and its poor resolution on the chromatogram. Methionine was expected to have a measurable intracellular pool for treatments containing the high concentration of this amino acid since methionine was entering the cell at a rate proportional to its concentration in the medium (Table 5). Because methionine was significantly metabolized upon entering the cell, there was no measurable intracellular pool even with those treatments containing elevated methionine. The lack of a measurable pool would suggest that the methionine requirement was not being met. Previous studies with dispersed cell culture using rat mammary tissue suggest that the optimum methionine for maximum milk protein synthesis was around 4.5 times the concentration in MEM and the treatments with the 5X methionine would be meeting the cell's requirement for this amino acid (Park et al., 1976a).

The intracellular lysine pool increased relative to its exogenous concentration (Table 6). Increased medium concentrations of lysine also resulted in an increase in the arginine pool. A possible explanation is that arginase, known to be present in rat mammary cells (Yip and Knox, 1972), is inhibited by increased lysine (Greenberg, 1960). This explanation does not appear to be consistent with the stimulatory

effect of high quantities of lysine on protein synthesis in rat mammary cells (Park et al., 1976a). Further work would be required to explain the lysine-arginine interaction.

Nonspecific Binding of Labeled Lysine and Leucine to Milk Protein

Table 8 gives calculated β -casein and β -lactoglobulin syntheses due to the binding of labeled lysine and leucine. The binding of lysine and leucine to skim milk was not significantly different. The values for protein synthesis due to nonspecific binding of labeled amino acids found in this study would account for approximately 25% of the milk protein synthesis reported by Schingoethe et al. (1967). This value was unexpectedly high compared to the 4% value reported previously by Groves and Larson (1965). While the calculated syntheses of β -casein and β -lactoglobulin significantly ($P < .05$) increased with the level of amino acids in the medium it was not at a uniform rate. From this study it was evident that nonspecific binding of labeled amino acid could introduce sizeable error.

Interaction Between Extracellular Lysine and Leucine with Labeled Lysine and Leucine in the Medium

Net β -lactoglobulin and β -casein syntheses as measured by incorporation of L-[U-¹⁴C] leucine or lysine are presented in Tables 9 and 10. Amino acid composition of the medium did not significantly affect β -casein or β -lactoglobulin synthesis. Average β -lactoglobulin as measured by L-[U-¹⁴C] leucine was 5.47 μ g/flask and 5.28 μ g/flask when measured with L-[U-¹⁴C] lysine. Synthesis of β -casein measured with L-[U-¹⁴C] leucine averaged 9.4 μ g/flask and measured with

Table 8. Calculated milk protein synthesis due to nonspecific binding of labeled amino acids^a

Medium Amino Acids	L-[U- ¹⁴ C]	β-casein (μg/flask)		β-lactoglobulin (μg/flask)	
		Lysine	Leucine	Lysine	Leucine
1-fold MEM		2.18	1.68	1.05	.89
3-fold MEM		3.94	3.49	1.92	1.52
5-fold MEM		6.93	5.44	3.79	2.15
7-fold MEM		8.29	8.87	3.73	3.58

^aData are means from 2 observations. Coefficient of variation for β-casein = 37% and for β-lactoglobulin = 23%.

Table 9. Effect of labeled amino acid on β -lactoglobulin measurement ^d

L-[U- ¹⁴ C] Amino Acid	Complement of Amino Acids in Medium		
	MEM + Leu	MEM + Lys	MEM
Lysine	4.35 ^A	7.95 ^B	3.55 ^C
Leucine	6.69 ^a	3.63 ^b	6.10 ^c

^{ABC} Orthogonal comparisons, means with upper case letters differ ($P < .05$) from means with lower case letters in same column.

^d Data are means from 6 observations expressed as $\mu\text{g}/\text{flask}$. Coefficient of variation = 40%.

Table 10. Effect of labeled amino acid on β -casein measurement ^d

L-[U- ¹⁴ C] Amino Acid	Complement of Amino Acids in Medium		
	MEM + Leu	MEM + Lys	MEM
Lysine	6.85 ^A	14.67 ^B	8.79 ^C
Leucine	10.56 ^a	6.89 ^b	10.80 ^c

^{ABC} Orthogonal comparisons, means with upper case letters differ (P<.05) from means with lower case letters in same column.

^d Data are means of 6 observations expressed as μ g/flask.
Coefficient of variation = 38%.

L-[U-¹⁴C] lysine averaged 10.10 µg/flask. There were no differences in measured protein due to labeled amino acid. The interaction between labeled amino acid and amino acid complement in the medium was significant ($P < .05$). Orthogonal comparisons made in Tables 9 and 10 show that labeled lysine significantly ($P < .05$) overestimated the effect of lysine concentration in the medium. A similar relationship was identified for labeled leucine and leucine concentration.

The overestimated effects of lysine and leucine are probably due to the nonspecific binding of labeled lysine and leucine similar to that observed in the previous experiment. When comparing effects of treatments on protein synthesis, nonspecifically bound labeled amino acid would be relatively constant across treatments as long as the specific activity of the labeled amino acid in the medium is the same. However, when the specific activity of the labeled amino acid is varied, the amount of labeled amino acid bound to the protein also changes. This introduces an error of varying magnitude in the calculated protein synthesis when treatments with different specific activities are compared. Based on these results, the use of labeled lysine to measure the effect of lysine in the medium or any other labeled amino acid to measure that amino acid's effect should be avoided. This probably accounts for part of the response to increased lysine in the medium observed by Park et al. (1976a).

Extracellular Amino Acid Effects on In Vitro Bovine Milk Protein Synthesis and Intracellular Amino Acid Pools

Approximately 47 percent of the mammary cells cultured were viable after the 18 hr incubation period (Table 11) which was a high culturing

Table 11. Response to graded amino acids in the medium

Block	Complement ^a amino acids	Percent Culturing ^a Efficiency	TCA Precipitate ^b μ gN/flask	TCA Soluble Nitrogen μ gN/flask	β-casein ^c μ g/10 ⁶ cells	β-lactoglobulin μ g/10 ⁶ cells
1	1-fold MEM	46.7	138	55	2.81	1.15
2	1-fold MEM	58.5	191	75	1.93	.75
1	3-fold MEM	46.0	141	66	6.17	2.06
2	3-fold MEM	46.2	79	40	2.30	.78
1	5-fold MEM	40.0	99	44	8.59	4.41
2	5-fold MEM	49.3	150	64	5.29	1.11
1	7-fold MEM	38.7	73	30	9.63	5.54
2	7-fold MEM	48.5	168	72	5.85	2.00

^aIn block 1 1.3×10^7 cells were added per flask prior to incubation and in block 2 1.5×10^7 cells were added.

^bTotal Nitrogen (Kjeldahl) Technicon AutoAnalyzer II Industrial Method No. 146/71A, Technicon Industrial Systems, Terrytown, N.Y. 10591.

^cData are means of 6 observations. Coefficient of variation for β-casein = 33% and for β-lactoglobulin = 49%.

efficiency for this procedure (Twarog and Larson, 1962). The TCA-soluble nitrogen averaged 30 percent of the total cell nitrogen. This value is high due to the method of harvesting the cells. Piez and Eagle (1958) encountered similar values for TCA-soluble nitrogen when they scraped cells from the culture vessel with the aid of a rubber policeman. However, Piez and Eagle were able to reduce the TCA-soluble nitrogen to 10% by precipitating cell protein without mechanically disturbing the cell layer. While the method of harvesting cells can result in considerable breakdown of cellular protein, Eagle et al. (1961) found the effect on free intracellular amino acid pools is negligible when the external concentration of amino acids are at the concentration used in this study.

Both β -lactoglobulin and β -casein syntheses increased significantly ($P < .05$) with increasing total amino acid concentration in the medium. The response in β -casein was greater than the response in β -lactoglobulin (Figure 1). Schingoethe et al. (1967) observed increases in both β -lactoglobulin and β -casein but only β -casein was increased significantly ($P < .05$). Numerous factors could contribute to the differences observed in β -lactoglobulin synthesis. Perhaps one of the more important factors might be the level of in vivo milk production by the mammary tissue cultured. Emery et al. (1970) observed that in vitro bovine milk protein synthesis was related to the animal's milk production prior to slaughter. The cows used in this experiment were producing more than 22 kg milk per day which was apparently greater than the production of the animals used by

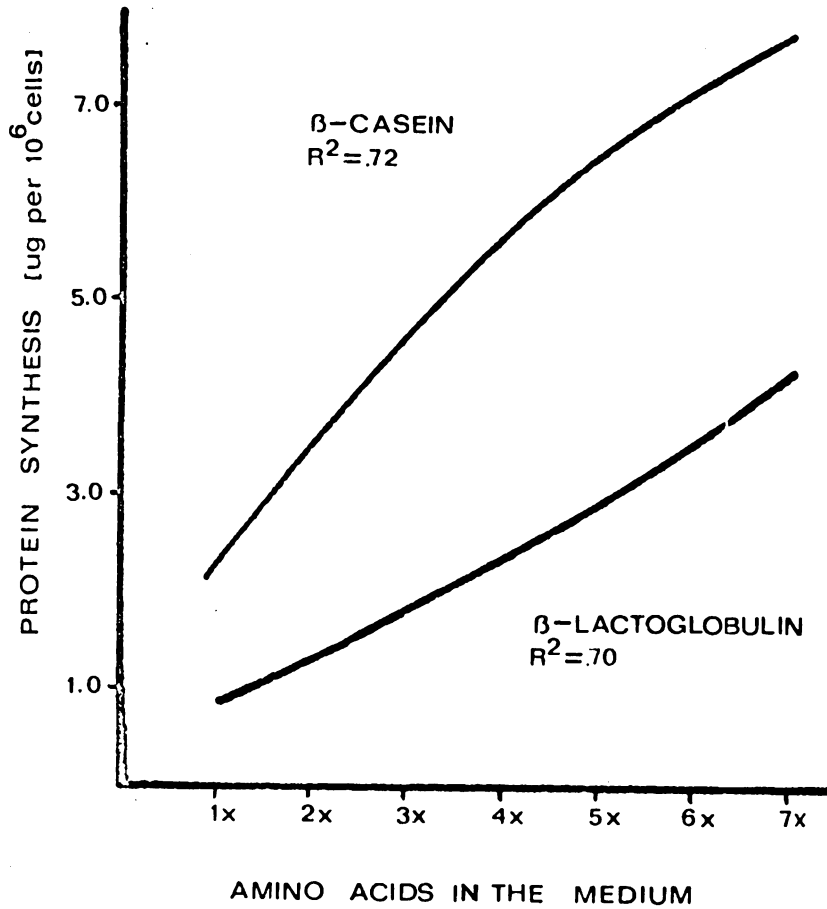


Figure 1. Effect of graded amino acid complement on milk protein synthesis by bovine mammary cells in culture.

Schingoethe et al. (1967). If this were the case, then the inherent ability of the mammary tissue of higher producing cows to respond to increased amino acids in the medium would be greater and could account for the significant response in β -lactoglobulin observed in this experiment. Both studies indicate that in culture, β -casein is more responsive to amino acid supplementation than β -lactoglobulin.

The mean values for the free intracellular amino acid pools are shown in Table 12. Glutamine is the only amino acid present in MEM which is not shown since it could not be quantitated satisfactorily. In addition to the amino acids present in MEM there were a number of nutritionally nonessential amino acids which were synthesized by the mammary cells. Of these serine, glycine, ornithine, alanine and glutamic acid were consistently present in amounts large enough to quantitate. As a group, these 6 amino acids contributed 49% of the total measured amino acid pool when the cells were cultured in MEM. As the concentration of the amino acids in the medium increased, the contribution of nonessential amino acids inside the cell decreased. Of the nonessential amino acids, glutamic acid was present in the largest amount. Regression equations for intracellular amino acids fitted by least squares analysis are given in the Appendix. The free intracellular nonessential amino acids are shown in Figure 2. Except for ornithine, the concentration of nonessential amino acids tend to decrease until the 5-fold concentration of MEM amino acids in the medium is reached, where the rate of decline begins to diminish. Intracellular ornithine concentration increases with extracellular amino acids until the 5-fold concentration of MEM amino acids is reached.

Table 12. Free amino acid pools in bovine mammary cells ^a

Amino Acid	Amino acid composition of medium				C.V. ^c
	1X	3X	5X	7X	
	----- μ moles/2x10 ⁶ cells-----				
Thr	3.03 ^b	5.73	7.14	8.94	23
Ser	1.93	1.32	.99	.94	23
Glu	6.84	4.62	3.37	2.85	23
Pro	2.83	1.84	1.40	1.31	24
Gly	5.16	2.91	2.61	2.50	30
Ala	3.55	2.54	1.99	1.81	27
Val	2.32	5.09	6.77	8.98	35
1/2Cys	.67	.90	1.35	1.82	38
Met	.08	.11	.13	.10	54
Ile	2.58	7.11	9.31	11.56	40
Leu	2.54	5.19	6.81	9.00	37
Tyr	1.29	2.76	3.58	4.90	32
Phe	1.68	3.53	4.44	5.62	36
Trp	.83	1.00	.70	.51	59
Orn	.24	.89	1.04	1.05	58
Lys	2.19	4.47	6.09	8.46	33
His	1.24	2.52	3.34	4.57	33
Arg	3.13	6.58	9.51	11.81	37

^a Amino Acid Analyzer Technicon - TSM system technical publication no. TAI-0233-10.

^b Each value is the mean of 4 observations.

^c Coefficient of variation.

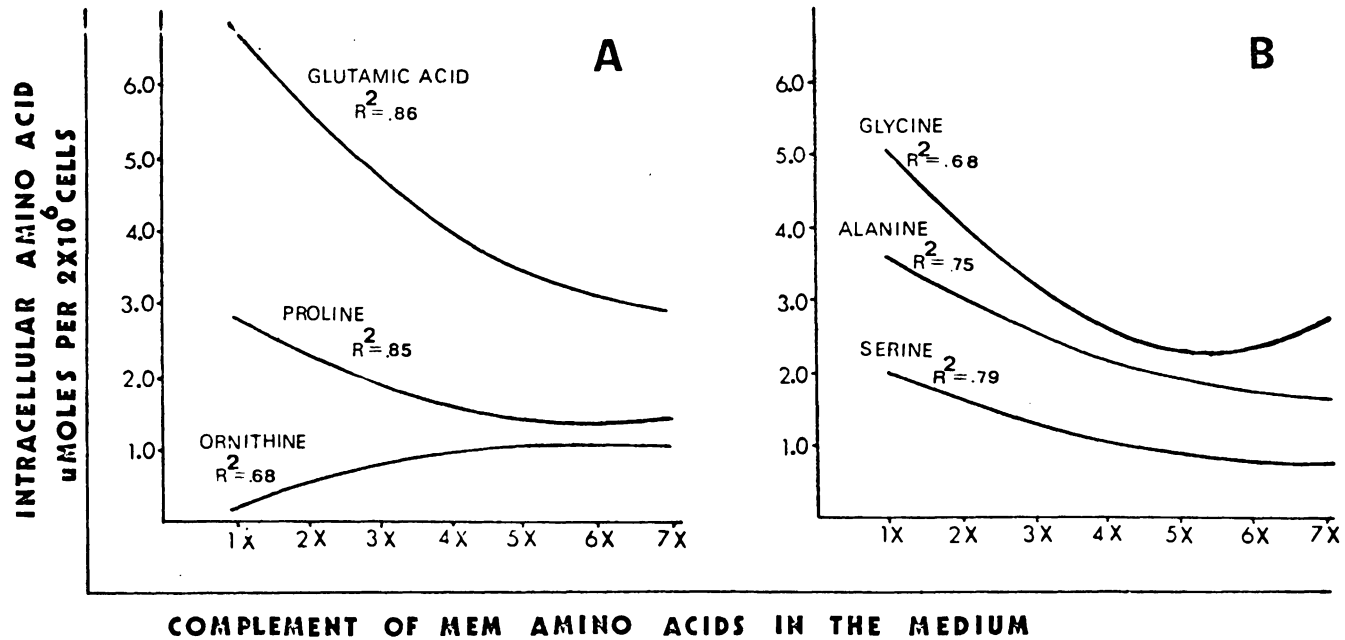


Figure 2. Effect of graded amino acid complement on nutritionally nonessential intracellular amino acids in bovine mammary cells.

The increase in ornithine observed in this experiment was probably due to the cleavage of arginine by arginase, an enzyme present in bovine mammary tissue (Clark et al., 1975). Arginine was one of the most abundant intracellular amino acids observed in this study. In studies measuring cell growth and using MEM as the medium, intracellular arginine is present in very small amounts (Piez and Eagle, 1958; Mohri, 1967). This suggests a difference in arginine required by growing cells and lactating mammary cells. While cystine did significantly ($P < .05$) increase with the level of amino acids in MEM, tryptophan and methionine did not respond to increased extracellular amino acids. Part of the difficulty in determining a response in tryptophan is that in analysis by the chromatograph a broad peak results which is poorly defined. This might partly explain the lack of an observed response in the tryptophan pool.

The methionine pool in several different types of mammalian cells in culture is very small (Kuchler, 1964; Mohri, 1967). As methionine in high concentration is toxic to cells, the presence of a sizable pool of this amino acid in mammalian cells may not be desirable. The lack of a sizable cystine pool was probably due to the formation of a mixed disulfide with glutathione. Acid hydrolysis of the TCA extract from human cells in culture resulted in significant increases in free cystine (Piez and Eagle, 1958).

A significant ($P < .05$) increase in intracellular lysine, histidine, tyrosine, leucine and valine was observed. Their increase was essentially linear with the extracellular amino acid concentration

(Figure 3). Phenylalanine, isoleucine, arginine and threonine pools in the cell also increased significantly ($P < .05$) with medium amino acids. However, their rate of accumulation at the 7-fold amino acid concentration was beginning to decrease, perhaps indicating saturation of the uptake of these amino acids (Figure 4).

The slopes of the regression equations shown in Figures 3 and 4 are an indication of the mammary cell's ability to concentrate medium amino acids intracellular. The internal concentration of the amino acids in Figures 3 and 4 increase with the external amino acids, but at their own characteristic rate. Isoleucine (Figure 4A) was the most efficiently accumulated amino acid by the mammary cell while methionine and tryptophan were the least efficiently accumulated. The lack of a response in the tryptophan and methionine pools to extracellular amino acids and the relatively small pool of cystine suggest that these amino acids may not be present in sufficient quantities for optimum functioning of the mammary cells.

Limiting Amino Acids for Milk Protein Synthesis by Bovine Mammary Cells in Culture

The results of adding single amino acids to the medium are shown in Table 13. The four amino acids which stimulated the most β -lactoglobulin, and β -casein syntheses were cystine, threonine, methionine and tryptophan. Threonine and methionine significantly ($P < .05$) increased β -lactoglobulin, while cystine significantly ($P < .05$) increased β -casein. Combined effect of amino acid addition on β -lactoglobulin and β -casein showed cystine to be the most limiting amino

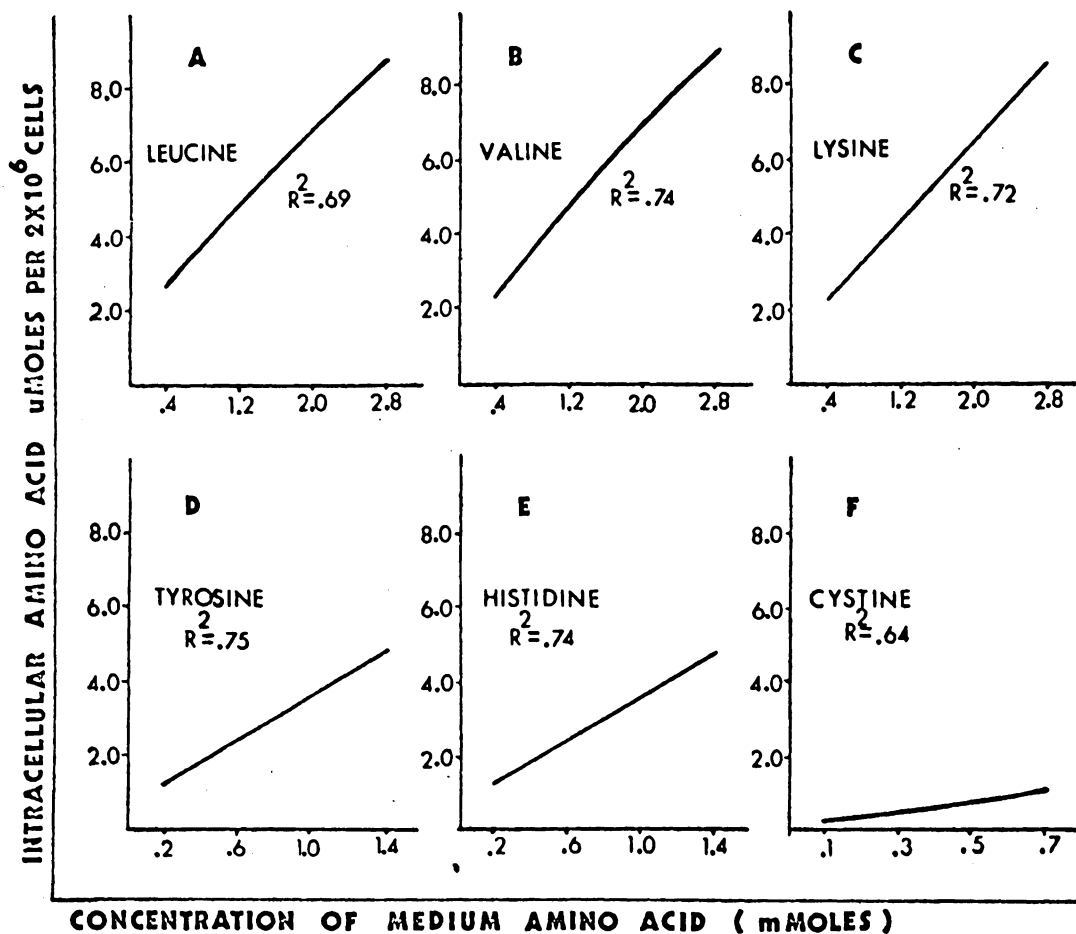


Figure 3. Intracellular amino acids with a linear response to increased extracellular amino acids.

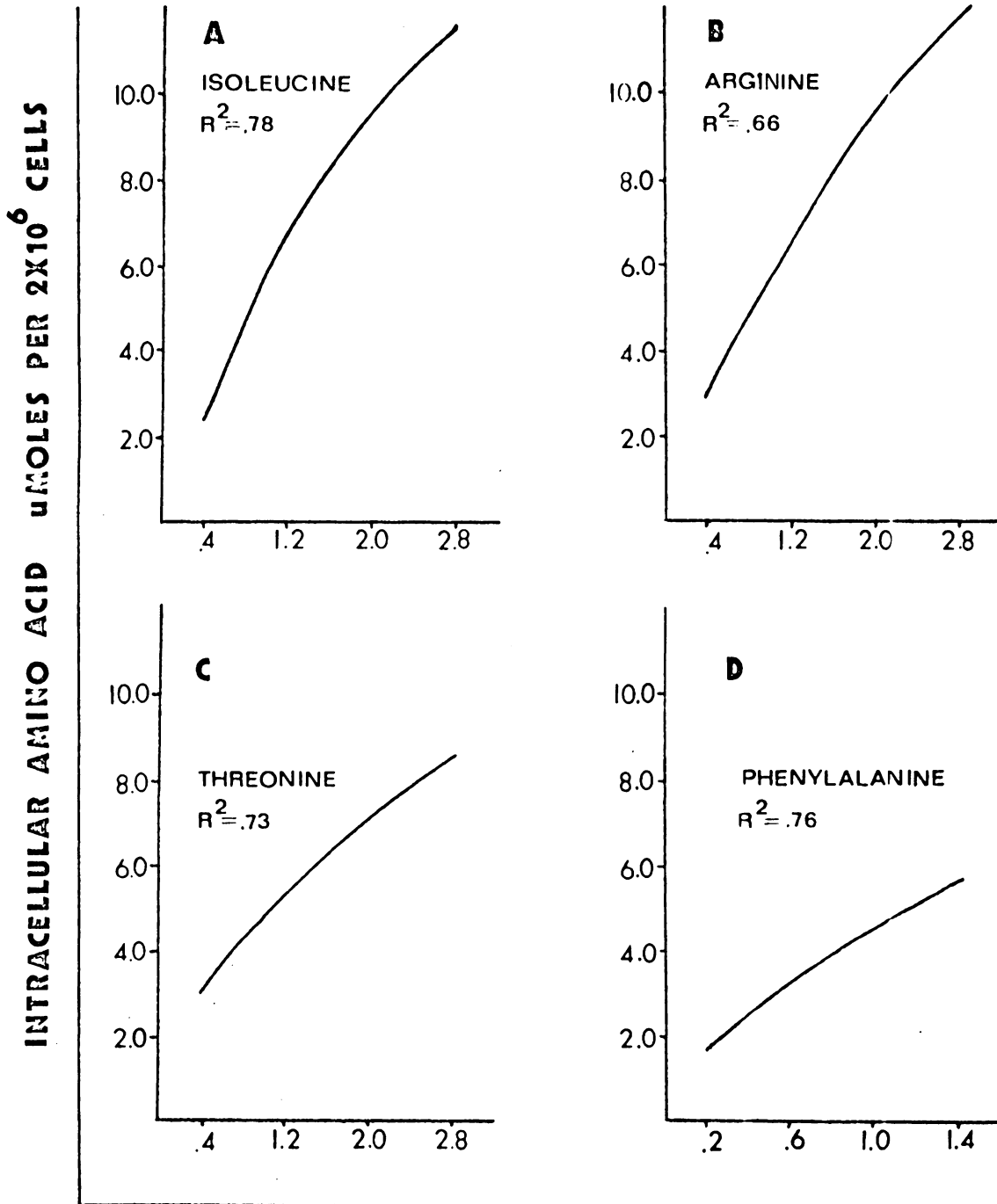


Figure 4. Intracellular amino acids beginning to show saturated uptake.

Table 13. Effect of amino acid addition on milk protein synthesis

β -Lactoglobulin μ g/flask	β -Casein μ g/flask	Combined Response L-value ^g
Thr ^f 7.28 ^a	Cys 14.20 ^a	Cys 19.24 ^a
Met 6.02 ^{ab}	Met 12.35 ^{ab}	Thr 18.76 ^{ab}
Trp 5.95 ^{abc}	Thr 11.93 ^{abc}	Met 18.00 ^{abc}
Cys 5.38 ^{abc}	Trp 11.87 ^{abcd}	Trp 17.46 ^{abcd}
Leu 4.85 ^{bc}	Arg 11.70 ^{abcd}	Arg 16.13 ^{abcde}
Arg 4.73 ^{bc}	Lys 11.49 ^{abcde}	Lys 15.42 ^{abcde}
Val 4.60 ^{bc}	Tyr 11.26 ^{bcde}	Phe 14.92 ^{bcde}
Lys 4.19 ^{bc}	Phe 11.14 ^{bcde}	His 14.63 ^{cde}
Phe 4.03 ^{bc}	His 11.09 ^{bcde}	Tyr 14.61 ^{cde}
His 3.78 ^{bc}	Ile 10.74 ^{bcde}	Ile 14.03 ^{de}
MEM 3.62 ^c	MEM 10.29 ^{bcde}	MEM 13.68 ^{de}
Tyr 3.57 ^c	Gln 9.04 ^{cde}	Val 13.06 ^e
Ile 3.51 ^c	Val 8.74 ^{de}	Leu 12.75 ^e
Gln 3.50 ^c	Leu 8.20 ^e	Gln 12.32 ^e
C.V.(%) 50.	25.	----

^{abcde} Means in same column not bearing a common superscript differ significantly (P<.05).

^f Amino acid elevated 3-fold in MEM.

^g Linear combination of means (12).

acid in MEM followed by threonine and then methionine. The difference in response to specific amino acids may be due to the difference in amino acid composition of the two proteins. The requirement for cystine, threonine and methionine for milk protein synthesis by bovine mammary cells in culture was demonstrated previously by Schingoethe et al. (1967). Work by Larson (1972) supports the response in β -lactoglobulin and β -casein syntheses to methionine as observed in the present study.

Considerable work has been conducted in vivo with methionine supplementation in lactating cows. Results of this experiment suggest that part of the action of methionine on protein synthesis might be through biosynthesis of cystine. Schingoethe et al. (1967) noted the synthesis of cystine from methionine may be limited in bovine mammary tissue since elevation of methionine did not affect the requirement for cystine. Eagle et al. (1961) observed in cell cultures with a low number of cells, cystine was required due to the leakage of intermediates for its synthesis from methionine. The number of cells cultured in this study were less than the number required for optimum lactose synthesis by bovine mammary cells in culture (Rao et al., 1975). As nutrients readily pass out of mammary cells, it must be considered that the response to cystine may be caused by a loss of intermediates for its synthesis. The ability of the mammary cell to convert methionine to cystine, in vivo and in vitro, requires further investigation before the significance of the cystine requirement can be explained.

The average synthesis of β -lactoglobulin was 4.60 $\mu\text{g}/\text{flask}$ and β -casein was 11.13 $\mu\text{g}/\text{flask}$. This is a lower rate of synthesis than

observed previously with rat mammary cells in similar experiments (Park et al., 1976b). The responses to single amino acid additions were also reduced when compared to the rat experiment. Emery et al. (1970) observed that in vitro synthesis of milk protein is proportional to previous milk synthesis in vivo. If this is true, then the low producing animals (ave. 12.7 kg milk/day) in this study have a limited ability to respond to increased nutrient availability.

Response to specific amino acids differed greatly among cows. β -lactoglobulin and β -casein often did not respond to the same amino acid within replications. Larson (1972) observed similar variability in milk protein synthesis in response to methionine stimulation of bovine mammary cells in culture. Mammary tissue from two cows in his study showed no response to methionine. Factors associated with mammary tissue that could cause variation are site of sampling, udder health, stage of lactation, milk production and degree of differentiation of mammary tissue. These are a few of the factors which could cause variability in response of bovine mammary cells in culture. Because of the relatively low production of the cows used in the present study, demands for nutrients might be reduced, allowing the nutrients present in the mammary tissue in vivo and carried into the culture to alter significantly the response to amino acids in the medium.

Eagle's minimal essential medium is a growth medium (Eagle, 1959). As the products of growth and milk synthesis differ one would expect the requirements to differ for each function of the cell. Results

suggest that the balance of amino acids in MEM could be improved for milk protein synthesis by increasing threonine and the sulfur containing amino acids.

Relative Uptake of Amino Acids and Their Requirement

In Table 14 the values for the combined response of β -lactoglobulin and β -casein syntheses determined in the single amino acid addition experiment, are presented along with the rate of change in individual amino acid pool concentration (1- to 3-fold MEM), from the bovine intracellular amino acid study. As the efficiency with which an amino acid's intracellular pool is maintained partially determines the cellular requirement for that amino acid, one would expect a decreased change in pool size with an increase in requirement for that amino acid. The correlation coefficient between milk protein synthesis in response to an added amino acid and change in that amino acid's pool size was $-.51$ ($P < .09$). Cystine, methionine and tryptophan had the smallest rate of change in pool size and when elevated in MEM were 3 of the top 4 amino acids to stimulate milk protein synthesis (Table 13). Threonine was determined to be the second limiting amino acid in MEM and had one of the greatest rates of intracellular pool size change (Table 14).

Eagle and Piez, 1962 and Kuchler, 1965 found threonine was concentrated to a much greater extent than other essential amino acids by mammalian cells in culture. In this work threonine did not appear as the most actively concentrated essential amino acid possible because of the mammary cell's requirement for it. If threonine were not considered,

Table 14. Amino acid stimulatory effect on milk protein and relative uptake

<u>Amino Acid</u>	<u>Milk Protein^a Synthesis</u>	<u>Relative Change^b in Pool Size</u>
Cystine	19.24	.135
Threonine	18.76	1.175
Methionine	18.00	.018
Tryptophan	17.46	.026
Arginine	16.13	1.736
Lysine	15.42	1.000
Phenylalanine	14.92	.802
Histidine	14.63	.553
Tyrosine	14.61	.621
Isoleucine	14.03	2.024
Valine	13.06	1.221
Leucine	12.75	1.164

^aL-values from Table 13.

^bChange in μ moles of intracellular amino acid per change in m moles of extracellular amino acid (Table 12 and Appendix Table 1).

the negative correlation between pool size change and milk protein synthesis would have been greater. The results of this study suggest the change in intracellular amino acid pool size might indicate amino acids which are not present in sufficient quantities in the medium, such as cystine, tryptophan and methionine. However, other factors controlling amino acid pool size limit its use as an indicator of cell amino acid requirement.

SUMMARY AND CONCLUSIONS

Rat mammary tissue, 2 to 5 days postpartum, was cultured with MEM, MEM with 5X concentration of lysine or methionine only, or MEM with 5X concentration of total amino acid complement. Intracellular amino acid pool size and uptakes of L-[U-¹⁴C] methionine and lysine were measured. Intracellular radioactivity indicated lysine and methionine concentrations inside the cell increased with increasing concentrations in the medium. Elevating all amino acids in MEM resulted in increased intracellular radioactivity from labeled methionine and lysine, but the increase was not as great as that obtained when methionine and lysine were elevated in the medium individually. Once methionine entered the mammary cell it was altered significantly. After culturing, only 42% of the intracellular radioactivity from labeled methionine was associated with intracellular methionine. Intracellular amino acids as measured by ion exchange chromatography showed tryptophan and methionine to be below the limit of detection. Increased medium lysine caused an increase in the intracellular arginine pool. As lysine freely enters the mammary cell, the high requirement reported by Park et al. (1976a) could not be explained by a restriction on its transport into the cell.

The binding of labeled amino acids to nonradioactive milk protein could cause significant error in calculated synthesis of milk protein when determined by the procedure of Groves and Larson (1965). This was particularly critical when the effect of an amino acid on protein synthesis was measured by the same amino acid serving as a labeled precursor for protein synthesis. When labeled precursor amino

acid and treatment amino acid were the same the response for that amino acid was overestimated. While the work of Schingoethe et al. (1967) would support a high requirement for lysine by the rat mammary cell, the extremely high requirement determined by Park et al. (1976a) could have been partially due to nonspecific binding of labeled lysine to carrier milk protein.

Mammary tissue from 2 Holstein cows was cultured with MEM containing graded concentrations of amino acids up to 7-fold the original concentration in MEM. Synthesis of β -casein and β -lactoglobulin, to a lesser degree, increased with the concentration of amino acids in the medium. Intracellular pools of nonessential amino acids not provided by MEM (serine, proline, alanine, glycine, and glutamine) decreased with increased concentrations of amino acids in the medium. Ornithine, which is also not present in MEM, increased to the 5-fold concentration of amino acids and plateaued thereafter. A linear increase in lysine, histidine, tyrosine, leucine and valine intracellular pools was observed whereas intracellular phenylalanine, isoleucine, arginine and threonine increased at a diminishing rate with increased medium amino acids. Intracellular cystine responded with a small linear increase to increased amino acids in the medium, while intracellular tryptophan and methionine did not respond, indicating potential limitations due to these amino acids.

To determine the effect of single amino acid addition on bovine milk protein synthesis in culture, mammary tissue from 10 Holstein cows was cultured in different amino acid complements. The treatments

consisted of a single amino acid elevation (3-fold) for each of the 13 amino acids in MEM. Increased threonine and methionine, significantly improved β -lactoglobulin synthesis over MEM, while cystine significantly increased β -casein ($P < .05$). Second and third best responses in β -casein synthesis were from methionine and threonine addition ($P > .05$). The responses to individual amino acid increases differed markedly among cows. Bovine mammary tissue did not appear as responsive to amino acid addition as rat mammary tissue in previous work (Park et al., 1976b).

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APPENDIX

Appendix Table 1. Least squares regression equations for intracellular amino acids present in MEM

Amino Acid	Regression Equation ^a	R ²	Sy.12	dy/dx at 2x ^b
Arginine	$Y = 1.172 + 2.024X - .072X^2$.66	2.86	1.736
1/2 Cystine	$Y = .569 + .075X + .015X^2$.64	.45	.135
Histidine	$Y = .723 + .565X - .003X^2$.74	.97	.553
Isoleucine	$Y = .250 + 2.591X - .142X^2$.78	3.07	2.024
Leucine	$Y = 1.368 + 1.278X - .028X^2$.69	2.19	1.164
Lysine	$Y = 1.272 + .977X + .006X^2$.72	.72	1.000
Methionine	$Y = .047 + .032X - .003X^2$.56	.06	.018
Phenylalanine	$Y = .815 + .968X - .041X^2$.76	1.36	.802
Threonine	$Y = 1.770 + 1.402X - .057X^2$.73	2.19	1.175
Tryptophan	$Y = .768 + .115X - .022X^2$.63	.45	.026
Tyrosine	$Y = .695 + .660X - .010X^2$.75	.99	.621
Valine	$Y = 1.080 + 1.359X - .035X^2$.74	2.03	1.221

^aIndependent variable X = millimoles of extracellular amino acid, dependent variable Y = micromoles of free intracellular amino acid.

^bTwo times the concentration in Eagle's minimal essential medium (Eagle, 1959).

Appendix Table 2. Least squares regression equations for intracellular amino acids not present in MEM

Amino Acid	Regression Equation ^{a,b}	R ²	Sy.12
Glutamic Acid	$Y = 8.236 - 1.515X + .107X^2$.86	1.02
Proline	$Y = 3.455 - .696X + .056X^2$.85	.45
Ornithine	$Y = -.157 + .454X - .041X^2$.68	.46
Glycine	$Y = 6.418 - 1.485X + .134X^2$.68	1.00
Alanine	$Y = 4.202 - .707X + .052X^2$.75	.66
Serine	$Y = 2.33 - .441X + .034X^2$.79	.30

^aIndependent variable X = multiples of the amino acid complement in Eagle's minimal essential medium (Eagle, 1959), dependent variable y = micromoles of free intracellular amino acid.

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EXTRACELLULAR AMINO ACID EFFECTS ON MILK PROTEIN
SYNTHESIS AND FREE AMINO ACID POOLS IN CULTURED
RAT AND BOVINE MAMMARY CELLS

by

Richard Martin Clark

(ABSTRACT)

Mammary cells from lactating rats and dairy cows were cultured in Eagle's minimal essential medium (MEM) with added amino acids. Changes in free intracellular amino acid pools and milk protein synthesis in response to amino acid additions to the medium were measured. Increases in free intracellular amino acid pools are associated with increased protein synthesis and the rate of their change in response to extracellular amino acids would partially reflect the cell's amino acid requirement. The intracellular pools from medium amino acids (except methionine, tryptophan and glutamine) increased with extracellular amino acids but at their own characteristic rate. The ratio of medium amino acids to nonmedium amino acids inside the mammary cell increased with the concentration of amino acids in the medium. Methionine and tryptophan did not have measurable pools in the rat mammary cell and only very small pools in the bovine mammary cell which did not increase with extracellular amino acids. Culturing rat mammary cells with labeled methionine showed only 42% of the intracellular radioactivity was still associated with labeled methionine indicating significant conversion of this amino acid after it entered the cell. A small linear increase in intracellular cystine was observed with elevated cystine in the medium. The responses in cystine, tryptophan and methionine intracellular pools

to extracellular amino acids suggest the concentration of these amino acids in the medium are insufficient to meet the bovine mammary cells requirement.

Increasing the concentration of amino acids in MEM 1-, 3-, 5- and 7 fold significantly ($P < .05$) increased β -casein and to a lesser degree β -lactoglobulin synthesis by bovine mammary cells in culture. Individually increasing each of the 13 amino acids in MEM 3 fold showed cystine followed by threonine and then methionine significantly ($P < .05$) increased the combined synthesis of β -casein and β -lactoglobulin. The correlation between intracellular pool size change and the response in milk protein synthesis to increased individual amino acids was $-.51$ which was not significant at the $.05$ level.