STUDIES ON (a) THE PRECURSORS OF MILK PROTEINS IN THE RABBIT AND (b) THE HORMONAL REQUIREMENTS OF GUINEA PIG MAMMARY TISSUE *IN VITRO*

Thesis for the Degree of Ph. D.
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George Contant Gerritsen
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This is to certify that the

thesis entitled

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PROTEINS IN THE RABBIT AND (b) THE
HORMONAL REQUIREMENTS OF GUINEA PIG
MAMMARY TISSUE *IN VITRO*

presented by

George Contant Gerritsen

has been accepted towards fulfillment
of the requirements for

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Joseph Meites
Major professor

Date 17 March 1960

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By

George Contant Gerritsen

AN ABSTRACT

Submitted to the School of Advanced Graduate Studies of
Michigan State University of Agriculture and
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Objectives

1. The objectives of this research were:

a. To provide quantitative data on the precursors of caseins, β-lactoglobulin and α-lactalbumin of rabbit milk.

b. To provide quantitative data on the relationship between γ-globulins of blood and immune globulins of rabbit milk.

c. To provide quantitative data on the relationship between albumin of blood and the "blood" albumin of rabbit milk.

d. To develop a synthetic medium enriched with hormones which would be capable of maintaining non-secretory mammary tissue *in vitro*.

e. To develop a medium enriched with hormones capable of initiation of lactation in non-secretory mammary tissue *in vitro*.

f. To develop a medium enriched with hormones capable of maintaining secretion in secretory mammary tissue *in vitro*.

g. To study the ability of secretory mammary tissue to take up amino acids and blood proteins *in vitro*.

Methods

1. The methods utilized in this research involved the blood fractionation by the procedures of Cohn *et al.* (1950). Albumins, γ-globulins and crude fractions of β-globulins and α-globulins were obtained by this method. Paper electrophoresis patterns were run on these fractions and on whole serum to prove their identity and establish their homogeneity.

2. Two male New Zeeland white rabbits were injected with C^{14}

labeled $BaCO_3$. The animals were placed in a chamber designed and built to trap expired C^{14} labeled CO_2. Six hours post injection, the animals were bled and C^{14} labeled serum protein fractions were isolated by the method of Cohn et al. (1950).

3. Classical ammonium sulfate fractionation procedures were employed for the fractionation of the β-lactoglobulin, α-lactalbumin and "blood" albumin in rabbit milk. Rabbit milk caseins were precipitated by adjusting the pH to their isoelectric point which was found to be 4.3. It was found that the immune globulin fraction of rabbit milk could be isolated by readjustment of the acid whey to pH 6.0. A method for paper electrophoresis of rabbit whey and rabbit milk proteins was developed which gave very good results. This method was the precoating of the paper strips with gelatin, thus preventing the absorption of the whey proteins into the paper strips.

4. The percent of leucine and glutamic acid were determined in the blood protein and milk protein fractions. This was accomplished by hydrolysis of the proteins and by column chromatography. The eluents from the column were subjected to paper chromatography as a purity check. Aliquots of eluents were treated with ninhydrin and the amount of amino acid determined colorimetrically. Free blood leucine and glutamic acid were isolated from the blood by column chromatography. The chemical purity of DL-leucine-2-C^{14} and DL-glutamic acid-2-C^{14} was established by paper chromatography and subsequent counting of the paper strips in a gas flow strip counter.

5. A method was developed for the milking of rabbits. This was

accomplished by injecting 1.0 I.U. of oxytocin into the marginal ear vein. A beveled glass tube was placed over the nipple immediately following the injection. The tube was connected to a reservoir for collection of milk which was in turn connected to a water aspirator. A negative pressure of 15 mm. of Hg. was maintained in the system. Vigorous massage of the mammary gland from the periphery toward the base of the nipple was applied together with periodic interruption of the suction by partial removal of the tube from the nipple. Blood samples were obtained from the rabbits by heart puncture into the left ventricle.

6. DL-leucine-2-C^{14}, DL-glutamic acid-2-C^{14} and C^{14}-protein fractions were dissolved in a minimal amount of phosphate buffer (pH 7.2) and injected by marginal ear vein into 10 lactating rabbits. Blood and milk samples were obtained at 2, 6, 12, 24, 36, 48 and 72 hours post injection. The blood and milk samples were fractionated into the various serum and milk protein fractions described above.

7. The blood and milk protein fractions were dialyzed against distilled water until free of reagents. The fractions were then lyophillised. Glutamic acid was isolated from the protein fractions by acid hydrolysis and precipitation of the chloride of glutamic acid. The protein, free glutamic acid and leucine samples were counted by β-liquid scintillation counting procedures.

Milk Protein Studies

1. In Experiment 1, 45 uc. of DL-leucine-2-C^{14} was injected into a lactating rabbit on the 14th day postpartum. The specific activity

of leucine was determined in: serum, γ-globulins, β-globulins, α-globulins, albumin, caseins, immune globulins, α-lactalbumin, β-lactoglobulin and "blood" serum albumin. The specific activity of free blood leucine decreased at an extremely rapid rate during the first 10 minutes post injection. The calculated value at zero time was 157.2 uc./mm. in the extracellular water. At 10 minutes, the experimentally determined value was 3.7 uc./mm. The equation for this decrease in the specific activity of free blood leucine is $A=A_oe^{-0.375t}$. The $t_{\frac{1}{2}}$ value is 1.85 min. and the rate constant is 0.375 $min.^{-1}$. After 10 minutes the rate constant changes very drastically to a value of -0.00169 $min.^{-1}$ with a $t_{\frac{1}{2}}$ of 409.5 min. A system to explain this finding has been postulated. On the basis of these data and the shape of the curve, it has been postulated that the leucine injected has been sequestered somewhere in the animal's body. Likely possibilities are the reticulo-endothelial system, the intracellular pool and plasma protein binding.

After two hours the leucine from this sequestered pool appears to return to the extracellular leucine pool at a rate slightly less than the rate of removal of free leucine from the extracellular pool. Thus, it appears that only a small proportion of the injected DL-leucine-2-C^{14} was available for synthesis into milk proteins at any particular time. Thus, an attempt was made to establish the parameters for leucine as a direct precursor of milk proteins. It appears that the curve for free blood leucine as determined in Experiment 1 represents the amount of free blood leucine available for incorporation into milk proteins.

2. The data in Experiment 1 show that the leucine incorporated into caseins, β-lactoglobulin and α-lactalbumin of rabbit milk are derived from the free blood leucine. The curves fit the criteria for precursor product relationships very well. Further, it takes an average of 4 hours for a free blood leucine molecule to be incorporated into a milk protein and transferred to the ducts ready for excretion as a milk constituent.

3. The ratios of the specific activities of blood γ-globulins and milk immune globulins in Experiment 1 show that at least 73.5% of the milk immune globulins were derived directly from the blood γ-globulins. The immune globulins of the milk were not synthesized within the mammary gland since the specific activities of leucine incorporated into the immune globulins of milk and the specific activities of caseins, β-lactoglobulins and α-lactalbumins are different.

4. It is also concluded from Experiment 1 that at least 77.4% of the "blood" serum albumin in rabbit milk is derived directly from the albumin of blood plasma. This is based on the ratios of the specific activity in these 2 protein fractions. The blood γ-globulins and albumin rapidly achieve equilibrium with the milk immune globulins and "blood" serum albumin of milk. The ratio of the specific activity of the immune globulin from milk to γ-globulin from blood at 2 hours post injection was 0.916 and the ratio of specific activity of "blood" serum albumin from milk to albumin from blood at 2 hours post injection was 0.900, thus demonstrating the rapid equilibrium between these blood proteins and their corresponding milk proteins.

5. Experiments 2, 3 and 4 with DL-leucine-2-C^{14} and DL-glutamic acid-2-C^{14} completely substantiate the results on the first experiment with DL-leucine-2-C^{14}. It is noteworthy that all of the glutamic acid incorporated into caseins, β-lactoglobulin and α-lactalbumin of the milk of lactating rabbits apparently comes directly from the free blood glutamic acid.

6. Experiments 5 and 6 were designed to evaluate the importance of plasma proteins as precursors of milk proteins by the injection of C^{14} labeled -plasma protein fractions into lactating rabbits. When C^{14} labeled γ-globulins were injected into 2 lactating rabbits, a close correlation was obtained between the specific activities of the γ-globulins and milk immune globulins. The time required to establish equilibrium between the blood and the milk fractions was slightly more than 2 hours in one animal and slightly more than 6 hours in the other animal. After equilibrium had been reached, it was calculated from the ratios of the specific activities of milk immune globulins to γ-globulins that 93.4% of the milk immune globulin in the milk of one animal and 98.2% of milk immune globulin in the milk of the other animal were derived from the γ-globulin in the plasmas.

7. When C^{14} labeled albumins were injected into 2 lactating rabbits in Experiments 7 and 8, the relationship between the plasma albumins and "blood" serum albumin in milk was found to be similar to that for γ-globulins and milk immune globulins. In this case, it took between 6 and 12 hours to establish an equilibrium between the blood plasma albumin and the "blood" serum albumin from milk. This was true in both animals.

After equilibrium had been established, it was calculated from the ratios of the specific activities of "blood" serum albumin in milk to the specific activities of albumin in plasma that 92.2% of the "blood" serum albumin in the milk of one animal and 98.2% of the "blood" serum albumin in the milk of the other animal were derived from the albumin in the plasma.

8. Experiments 9 and 10 on the injection of C^{14} labeled α- and β-globulins from blood plasma indicated that they were of little importance as precursors of milk proteins. Also, no milk protein fraction was detected which was derived directly from these two blood protein fractions.

In Vitro Studies with Mammary Tissue Cultures

1. Mammary tissue was cultured by the organ culture method. The explants were placed on treated rafts of cellulose-acetate. The rafts were floated in the synthetic medium in a watch glass in a Petri dish. The explants were cultured for 5 days. The medium was changed when the pH dropped below 7.0. Histological sections of the explants were prepared and stained with iron hemotoxylin and eosin in the usual manner.

2. Media for mammary tissue culture were prepared from Parker's "199" synthetic medium. Prolactin, hydrocortisone and insulin were added at 2 concentration levels. The levels of these hormones for mammary tissue maintenance were 140, 8 and 70 µg./ml. of medium, respectively. The levels of these hormones for maintenance of secretion in mammary tissue were 240, 16 and 140 µg./ml. of medium, respectively. The media were gassed with 95% O_2 and 5% CO_2. DL-leucine-2-C^{14},

DL-glutamic acid-2-C^{14}, C^{14}- γ-globulin and C^{14}-albumin were also added to portions of the media for maintenance of secretion in vitro.

3. Autoradiographs were prepared from explants cultured in media containing C^{14} labeled amino acids or proteins. The sections of the tissues were mounted on microscope slides and stained with hemotoxylin and eosin in the usual way. The slides were coverslipped with celloidin. A thin film of Eastman Kodak emulsion was applied in the darkroom. The slides were sealed in a light proof container and placed in the refrigerator for exposure. The exposed autoradiographs were developed with dektal, fixed in acid fix and washed with tap water. They were then dehydrated and coverslipped in the usual manner.

4. It was shown that γ-globulin can be taken up by mammary tissue in active secretion and concentrated in the alveolar cells in vitro. This ability is destroyed by heating the explant to 50°C just prior to cultivation.

5. Prolactin (240 µg./ml.), hydrocortisone (16 µg./ml.) and insulin (140 µg./ml.) added to a synthetic medium are capable of maintaining active secretion in mammary tissue in vitro. This was shown by histological preparations and by the active uptake of leucine and glutamic acid and their apparent incorporation into mammary secretory products.

6. Albumin is not taken up by the secretory mammary tissue in vitro.

7. Heating explants to 50°C for 1 minute destroys the ability of mammary tissues to take up γ-globulins, leucine and glutamic acid in vitro.

8. Prolactin (140 µg./ml.), hydrocortisone (8 µg./ml.) and insulin (70 µg./ml.) in a synthetic medium are capable of maintaining non-secretory mammary tissue *in vitro*. Seventy-three percent of the total lobulo-alveolar tissue was maintained in 108 explants prepared from 8 non-lactating guinea pigs.

9. Prolactin (240 µg./ml.), hydrocortisone (16 µg./ml.) and insulin (70 µg./ml.) in a synthetic medium are capable of maintaining secretory mammary tissue *in vitro*. Seventy percent of the total lobulo-alveolar tissue was maintained, and 55.4% of the total lobulo-alveolar system was maintained in an active secretory state in 180 explants prepared from 12 lactating guinea pigs.

10. The data obtained on milk immune globulins and "blood" serum albumin in milk and the data obtained by the addition of C^{14} labeled γ-globulins and albumins to tissue culture media indicate that there is a different mechanism by which the mammary epithelium incorporates these proteins into milk. It is postulated that the mammary gland actively takes up -globulins or is permeable to -globulins, and that it does not actively take up plasma albumin or that the mammary gland is relatively impermeable to albumin.

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Dedicated

to my lovely, patient wife Marian

and son Steven

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INTRODUCTION

The biosynthesis of proteins is an extremely complex process. The synthesis of native proteins *in vitro* has never been accomplished, although brilliant investigations have led to the synthesis of peptides such as those found in insulin. The problem of protein synthesis has fascinated scientists for many years and has stimulated a tremendous amount of research in this area.

The mammary gland in full lactation is one of the most active synthesizers of proteins in the animal body. Milk is essential for the nutrition of the newborn and is one of the most important sources of high quality protein, readily available as a nutrient for human nutrition. Since the mammary gland lies outside of the body cavity, is readily accessible, and has a rapid rate of protein synthesis, it is an excellent organ for the study of the precursors of proteins.

The precursors of proteins in the milk have been studied by the following techniques: (1) arterio-venous differences (2) perfusion experiments (3) comparisons of electrophoretic mobilities and amino acid composition of blood and milk proteins (4) most recently, by isotope techniques. The data from these experiments have provided a great deal of information of a qualitative nature concerning the precursors of milk proteins. However, there is a decided lack of quantitative information concerning the precursors of milk protein. Also, a considerable amount of the literature on the precursors is conflicting. This is especially true of the early work on arterio-venous studies.

This thesis is mainly concerned with an attempt to throw additional light upon the problem of milk protein precursors in the rabbit by providing: (1) quantitative information on the role of free blood leucine and glutamic acid as precursors of milk proteins (2) quantitative information on the role of blood γ-globulins as the source of immune globulins in rabbit milk and (3) quantitative information on the source of "blood" serum albumin in rabbit milk. Radioactive leucine and glutamic acid were chosen for these investigations because of their relatively high concentration in milk proteins. Also, leucine is an essential amino acid and glutamic acid is an extremely labile non-essential amino acid.

The cost of radioactive amino acids is extremely high. Therefore, a decision had to be made concerning the animal in which the investigation was to be carried out. The decision was primarily whether to give one large dose to one larger animal, thus obtaining one set of large samples to work with, or to give a number of small doses to a small animal such as the rabbit and work with small samples. Statistically, there are disadvantages to either choice. The latter choice was made for these investigations.

There is very little information available on the role of serum proteins as precursors of milk proteins. For this reason, it was felt that these proteins should be evaluated as a possible source of milk proteins. An attempt was made to evaluate the role of blood protein fractions by labeling them with C^{14} and injecting the fractions into lactating rabbits with the subsequent isolation of the milk proteins.

Recently, attention has turned to the <u>in vitro</u> cultivation of mammary tissue. It was felt that this would be a good tool for the investigation of the uptake of amino acids and proteins by the mammary gland, since the gland is removed from the influences of other organs and the animal body in general. Thus, a large number of variables

can be controlled to a great extent. Experiments were therefore conducted to develop satisfactory media for the maintenance of guinea pig mammary explants *in vitro*.

It is hoped that the results of these experiments have helped to clarify the role of certain blood precursors in the synthesis of rabbit milk proteins and the *in vitro* hormonal requirements of guinea pig mammary tissue. However, it is felt that additional experiments must be done before these problems can be completely resolved.

REVIEW OF LITERATURE

I. The Biosynthesis of Milk Proteins

A. Techniques Utilized for the Study of Milk Protein Precursors and the Results Obtained

It is necessary to clarify the nomenclature of the various milk proteins referred to in this review of the literature on the precursors of milk proteins, since the same terminology is not employed by all workers. Jenness *et al.* (1956) have clarified the rather confusing nomenclature of the milk proteins which exists in the earlier literature. They have defined the milk proteins of the cow in the following manner:

1) Caseins: 80% of milk protein, molecular weight of 24,100, isoelectric point 4.7, mobility at pH 8.6 is -6.7.
2) β-Lactoglobulin: 7-12% of milk protein, molecular weight of 35,500, isoelectric point 5.18, mobility at pH 8.6 is -5.1.
3) α-Lactalbumin: 2-5% of milk protein, molecular weight of 15,100, isoelectric point 4.1-4.8, mobility at pH 8.6 is -4.2.
4) "Blood" Serum Albumin: 0.7-1.3% of milk protein, molecular weight of 65,000, isoelectric point 4.7, mobility at pH 8.6 is -6.7.
5) Immune Globulins (composed of two fractions):
   a) Euglobulins: 0.8-1.7% of milk protein, molecular weight of 180,000, isoelectric point of 6.0, mobility at pH 8.6 is -1.8.
   b) Pseudoglobulins: 0.6-1.4% of milk protein, molecular weight of 180,000, isoelectric point 5.6, mobility at pH 8.6 is -2.0-2.22.

This terminology will be strictly adhered to throughout the text.

1. Arterio-Venous Differences in the Concentrations of Amino Acids and Proteins of Blood

It is obvious that the secretory products of the mammary gland are originally derived from the blood passing through this gland. The basic problem is--what are the components of the blood which are the direct precursors of the milk proteins? Folley (1956) has stated that there are four main possibilities to consider in respect to the mechanism involved in the biosynthesis of milk proteins. Milk protein may be synthesized (1) entirely from the amino acids of the circulating blood (2) partly from the preceding and partly from amino acids arising from the degradation of blood plasma protein in the mammary gland (3) from blood plasma proteins by rearrangement of peptide chains involving transpeptidation reactions and (4) they may be derived only partly from the latter and partly from blood amino acids.

Arterio-venous studies can provide useful information about the uptake of a particular blood constituent by an organ. Since the mammary gland is outside of the body cavity, it is relatively easy to study the uptake of substances by the mammary gland from the blood by the arterio-venous technique. Cary (1920) was the first to study the uptake of amino acids by the mammary gland. He took blood samples simultaneously from the jugular and mammary (abdominal subcutaneous) veins of milking cows and determined the free amino nitrogen in the blood samples. He found that the blood from the mammary veins contained 16-34% less amino acid nitrogen than the jugular blood. Blood samples from dry cows gave values of -3 and 5% for amino acid differences and thus no uptake by the mammary gland. From these data he concluded that the amino acids removed from the blood by the mammary gland are sufficient to account for the proteins of milk and that they were undoubtedly the precursors of these milk constituents.

The work of Cary (1920) was repeated by Blackwood (1932). She took true arterial blood samples instead of jugular blood samples. Her arterio-venous differences for free amino nitrogen were between 8 and 15% on 4 lactating cows and 2 and -6% on 4 dry cows. This work also demonstrated uptake of amino acids by the secretory mammary gland.

Graham (1937) attempted to compare the quantity of free amino nitrogen absorbed from the blood by the udder of the lactating goat during the production of a given quantity of milk. He measured the blood flow through the udder with a flow meter in the mammary vein and the arterio-venous difference of free amino nitrogen was determined. The two goats were milked dry just prior to and at the conclusion of the experiment. His data indicated that all of the amino nitrogen secreted in the form of milk protein could not be accounted for by the free amino acids absorbed from the blood by the mammary gland. Studies with the arterio-venous technique by Lintzel (1934), Shaw and Petersen (1938), and Reineke _et al_. (1939) have led to the same conclusion as Graham (1937) that the uptake of amino acids by the mammary gland is not sufficient to account for all of the amino acids secreted in the form of milk proteins. Shaw and Petersen (1939a,b) stated that the amino acid uptake could not account for more than 40 to 50% of the casein nitrogen of milk.

It was principally because of the general acceptance that free blood amino acids could not account for all of the milk protein that attention was directed to the possibility of plasma proteins as milk protein precursors. Reineke _et al_. (1941) carried out arterio-venous studies which led them to the conclusion that some fraction of the plasma protein was utilized by the udder for the formation of milk protein. They postulated that this fraction was probably a globulin fraction containing protein-bound carbohydrate. Graham _et al_. (1938) also reported that a globulin of the blood was taken up by arterio-venous studies and utilized by the mammary gland. More recently, Nikitin (1949) has done arterio-venous studies and has reported that not more than 45% of the amino nitrogen of milk proteins comes from free amino nitrogen in the blood. His work is in general agreement with the earlier work.

The arterio-venous technique is subject to a number of errors which may lead to erroneous conclusions. Folley (1940, 1949) has critically reviewed the work done on the arterio-venous technique as a tool for measuring the blood precursors of milk constituents. In order to quantitate data of this nature, it is necessary to determine the volume of blood that has passed through the udder during the time that the milk is being synthesized. It is relatively easy to determine the amount of milk synthesized in a given period of time. However, the flow of blood is subject to large variations. There are two methods used to determine the blood flow through the udder. Graham _et al_. (1938) measured the blood flow directly with a flow meter and found that 150-250 volumes of blood passed through the goats udder for each volume of milk produced. The other method is based on the arterio-venous difference of calcium or phosphorus and the blood volume calculated from this value. The calculated values reported in the literature vary from 387 to 563. Folley (1949) believes the true value to be in the range of 400-600 volumes of blood to one volume of milk. Values based on calcium uptake would be in error since all of the calcium would not be secreted via the milk. Some would be returned via the lymph. Shaw and Petersen (1940) have reported that the flow of lymph from the mammary gland of the cow is considerable. Some calcium would undoubtedly go with it and cause a low blood to milk volume ratio by this method of calculation. The arterio-venous differences reported in the literature represent uptakes of averaged values over the relatively short periods of time required to collect the blood samples, and thus do not really represent the average value during the entire period of the experiment. The secretion

rate of milk is determined over a period of hours, during which the rates of blood flow and milk secretion may independently undergo considerable variation.

There is the possibility of systemic variations in the uptake of certain milk constituents from one milking to the next. This has been reported by Shaw and Petersen (1940). This could cause a very large error in arterio-venous studies. The arterio-venous differences are also subject to uncertainties arising from the possibility of their loss via the lymph and also from random or systematic changes in their rate of uptake as stated by Graham _et al_. (1936).

Shaw and Petersen (1939) observed appreciable blood volume changes when the subject was disturbed by blood sampling procedures. This appears to be due to vasomotor effects. In order to minimize vasomotor disturbances during blood sampling, Reineke _et al_. (1941a) proposed the use of nembutal to anaesthetize the animal. They reported that anaesthetized goats continued to secrete milk of normal composition at an unchanged rate. Shaw (1946) has also reported this to be true in cows anaesthetized with nembutal.

Barry (1958) has strongly criticized the arterio-venous technique. However, Sheldon-Peters and Barry (1956) published results obtained by the arterio-venous technique in which they state that all of the essential amino acids in casein were derived directly from the blood. Folley (1949) states, that in view of the considerations discussed above, it seems justifiable to conclude that quantitative balance experiments conducted on the mammary gland by the arterio-venous technique have very little validity; the arterio-venous technique in its present form is capable of giving qualitative results only.

2. Perfusion and Tissue Slice Studies on Mammary Glands

The first perfusion study was done by Foa (1912) on an isolated sheep's udder. Petersen *et al.* (1939, 1941) developed a practical apparatus for the perfusion of the bovine udder which eliminated many of the difficulties encountered in the arterio-venous technique. These advantages are that the general disturbances to the animal are eliminated and that the actual flow of blood and lymph may be measured at all stages of the experiment. Also, the metabolites can be added to the perfusate and their fate can be studied. This technique can also be used to detect precursors which are taken up by the mammary gland in small amounts, since the blood can be recirculated to increase the concentration difference. Petersen *et al.* stated that the gland functioned normally as evidenced by the disappearance of metabolites and normal uniform R.Q. values. Peeters and Massart (1947, 1952) also developed a perfusion technique for the udder independently of Petersen (1941).

The major disadvantage to this procedure is the difficulty of maintaining the optional levels of metabolites and hormones as pointed out by Reineke *et al.* (1941). It appears that the maintenance of the proper pH and the accumulation of metabolic waste products might also be a problem.

Bouckaert *et al.* (1953) have utilized the perfusion technique on the isolated udder of the cow to study the uptake of free amino acids. The perfused blood was passed through the isolated udder a total of 10 times and they found a significant decrease in 10 of the free amino acids of the blood. The uptakes were usually most marked in those amino acids which are found in high concentrations in casein. Casein hydrolysate was added to the blood perfused through one-half of the udder. The uptake in this half was higher than in the half that was perfused with blood that had no casein hydrolysate added. The work of Bouckaert *et al.* (1953) tends to indicate that mammary tissue selectively takes up the amino acids which are present in casein in the greatest concentration. Recently, Peeters *et al.* (1957) have carried out a perfusion experiment on a lactating cow's udder with the addition of 0.5 uc. of cysteine-S^{35} added to the perfusion blood. Their results are very interesting since they found twice as much activity in the whey proteins as in casein or plasma proteins at the end of the perfusion. Lauryssens *et al.* (1957) and Peeters *et al.* (1957) have perfused the cow udder with propionate-C^{14} and glucose-C^{14}. They found that the milk proteins were active when propionate-C^{14} was added to the perfusion blood.

Barry (1958) states in his review that McNaught and Folley (1958) have been able to demonstrate the uptake of radioactive amino acids by incubating mammary tissue slices. Also, Peeters *et al.* (1957) incubated bovine mammary tissue *in vitro* and found that the milk proteins were very slightly labeled. Tissue slices provide a useful technique for the study of precursors. However, one difficulty with this procedure is the estimation of the dry weight of the tissue, since variable amounts of milk will be retained in the tissue. This problem has been discussed by Folley and Greenbaum (1947).

The information obtained by perfusion and tissue slice techniques indicates that the mammary gland does take up and utilize free amino acids for the synthesis of milk proteins. The data provided by this work is of a qualitative rather than a quantitative nature.

3. The Use of Isotopes for the Investigation of the Precursors of Milk Proteins

The first application of labeled amino acids to the problem of milk protein precursors was by Campbell and Work (1952). They injected valine, lysine, and glycine labeled with C^{14} into a lactating rabbit and were able to show qualitatively that the greatest activity was present in casein and β-lactoglobulin. Barry (1952) carried out similar experiments on the lactating goat. He injected lysine and tyrosine intravenously and found high levels of activity in the casein. Askonas et al. (1954) injected radioactive glycine, valine, lysine and methionine into a lactating goat and found high specific activities of the amino acids in the caseins and β-lactoglobulin isolated from the milk.

Askonas et al. (1955) injected C^{14} labeled amino acids into a goat. They subsequently isolated the caseins and β-lactoglobulin from the animal's milk and subjected these protein fractions to partial hydrolysis. They separated specific peptides by column chromatography. The specific activities of these peptides were very similar. Their work indicates that casein and β-lactoglobulin were synthesized from free amino acids and not peptides, since the labeling in the protein chain was uniform. Barry (1956) injected labeled glutamic acid into a lactating goat. He reported that 50% of glutamic acid in the casein came directly from the free glutamic acid in the blood.

Larson and Gillespie (1957) injected a large dose of C^{14} labeled bicarbonate into a lactating cow. A very careful analysis of the milk proteins indicated a high level of activity in α- and β-casein, β-lactoglobulin and α-lactalbumin. The activity in these protein fractions was about equal, which suggested that they were all derived from the same amino acid pool. They reported low activities in the γ-casein, immune globulin fraction and "blood" serum albumin of the milk which indicated that their precursors were different. Larson and Gillespie (1957) suggested that the protein fractions of high specific activity were derived directly from the free blood amino acids.

Kleiber et al. (1952) found the same type of labeling when they injected labeled carbonate into the lactating cow.

Barry (1958) and Sansom and Barry (1958) have reported that most of the lysine, tyrosine, glutamine, glutamic acid, asparagine and proline incorporated into the caseins of goat's milk come directly from the corresponding free amino acids of the blood. They stated that 70% of essential and 50% of the non-essential amino acids incorporated into casein by synthesis in the goat's mammary gland come directly from the free amino acids of the blood.

Peeters et al. (1957) injected S^{35} cysteine into a lactating sheep. They found that the specific activity in the isolated whey proteins was much higher than the specific activity in the casein. The same result was obtained when S^{35} cysteine was added to the blood during perfusion experiments. This is interesting because it is just opposite to the results obtained by other workers as reported earlier in this section.

A review of the literature on the incorporation of labeled amino acids indicates that almost all of the work reported is of a qualitative nature. It is felt that there is a need for a quantitative investigation on the biosynthesis of milk proteins.

4. The Source of Immune Globulins and "Blood" Serum Albumins in Milk

The literature on the immune globulins in milk is voluminous and beyond the scope of this review. However, a few of the reports in the literature should be mentioned.

Ehrlich (1892) originally found that the colostrum from immune mice transmitted specific antibodies that were absorbed by the nursling. Crowther and Raistrick (1916) reported that there were proteins of a similar nature in both blood and colostrum. Howe (1921) was the first to show that the newborn calf acquires specific proteins upon ingestion of colostrum.

There have been a great many reports confirming these early findings. Smith (1946a, 1946b, 1946c, 1946d, 1957) has shown marked similarity between the γ-globulins of the blood and the immune globulins of milk and colostrum by amino acid composition and electrophoretic comparisons. However, he found some differences in amino acid composition. Hansen and Phillips (1947) and Hansen et al. (1947) have also shown the similarities between these two protein fractions. There are many other reports in the literature supporting the above conclusions. It has been shown many times that the blood of the newborn calf is deficient in γ-globulins and that after the ingestion of colostrum, γ-globulins appear very rapidly in the blood of these calves.

Recently, Larson (1958) has shown by quantitative electrophoresis that 85 to 107% of the immune globulins of bovine colostrum appear to come from the blood β_2- and γ_1-globulins. Askonas et al. (1954) immunized a lactating rabbit and then injected S^{35} labeled DL-methionine. The antibodies from milk and blood were precipitated by the antigen and counted. The counts were from 7-17 counts per minute. The activities in the milk and blood were about the same, but the activities are so low that there is some question about their significance. The previous work certainly indicates a relationship between the blood γ-globulins and immune globulins in the milk and colostrum. However, the evidence does not preclude the possibility that these γ-globulins could have been altered slightly within the mammary gland.

Polis et al. (1950) and Coulson and Stevens (1950) have reported "blood" serum albumin in milk and have indicated that it is very similar to albumin in blood. Larson and Gillespie (1957) have shown by quantitative electrophoresis that these two proteins are identical. There are no data in the literature on "blood" serum albumin in rabbit's milk, and it has been investigated in the present study.

5. *In Vitro* Cultivation of Mammary Tissue

The cultivation of mammary tissue *in vitro* has been one of the more difficult problems in the area of tissue culture. It has not been until recent years that progress has been made in this area. Hardy (1950) was the first to report the culture of mammary tissue. He reported duct growth but no alveolar development in organ cultures prepared from the ventral body wall of embryonic mice.

Lasfargues (1957a, 1957b) has reported proliferation of mammary epithelial cells after the dispersion of the mammary tissue cells by incubation with collagenase. He used various types of serum as a medium.

Elias (1957, 1959) and Elias and Rivera (1959) have reported cultivation of organ cultures from adult mice. They prepared their explants from fully developed mammary tissue of pregnant mice. The tissues were cultured in a synthetic medium enriched with various hormonal combinations at different levels. They found that the addition of estrogen, progesterone, cortisol, growth hormone and prolactin to the media maintained the mammary explants. Their work was the first successful demonstration of the organ culture of mammary tissue. These workers were also able to initiate some secretion in their explants by means of high levels of cortisol and prolactin. Trowell (1959) has reported the successful organ type cultivation of rat mammary tissue *in vitro*.

Larson (1959), Ebner and Larson (1958, 1959), and Hoover *et al.* (1959) have published abstracts on the cultivation of bovine mammary tissue *in vitro*. They have been able to grow sheets of mammary

epithelium and maintain this epithelium in culture for many months. Their work is extremely important because of indications of biosynthesis of lactose and β-lactoglobulin by these epithelial cells in culture. Their work indicates the potential of tissue culture techniques for biochemical investigations into the synthetic mechanisms of the mammary gland. Tissue culture experiments on mammary tissue are considered to be important because they provide *in vitro* techniques for studies of the biochemistry, endocrinology, and physiology of the mammary gland. This technique also provides a method for the study of the precursors of milk constituents.

MATERIALS AND METHODS

A. Methods Utilized in the Studies of Rabbit Milk Protein Precursors

1. Determination of the Radio-Chemical Purity of Leucine-2-C^{14} and Glutamic Acid-2-C^{14}

The radio-chemical purity of Leucine-2-C^{14} was ascertained by paper chromatography of a small sample of the material which was purchased from Tracerlab, Inc., 130 High Street, Boston 10, Massachusetts. The amino acid was chromatographed on Whatman No. 1 paper by the descending technique. The solvent was 88% liquid phenol:2-propanol:water = 100:20:20 v/V. The glutamic acid-2-C^{14} was purchased from the same company and chromatographed in the same manner. The solvent used was N-Butyl alcohol:acetic acid:water = 250:60:250 v/V. The detailed procedures are given by Block and Weiss (1956a).

The above chromatographs showed a single spot with corresponding R_f values in the proper range. Also, the radioactivity of these strips was located over the individual spots. These determinations were made by means of a gas flow strip counter at the Argonne National Laboratories, Lemont, Illinois. Figure 1 in the appendix shows a photograph of these chromatographs together with the recording of radioactivity.

The specific activities of the leucine-2-C^{14} and glutamic acid-2-C^{14} were 0.51 mc./mm. and 0.54 mc./mm., respectively. Thus, it was established that the materials to be injected were pure and that the resultant activity in isolated proteins was derived from the injected amino acid.

2. The Production of C^{14} Labeled Rabbit Serum Proteins and Their Fractionation

These proteins were produced *in vivo* by the injection of a dose of 11.85 mg. of $BaCO_3$-C^{14} (1.5 mc.) into the marginal ear vein of each of two male New Zealand white rabbits. The C^{14} labeled $BaCO_3$ was purchased from the Union Carbide Nuclear Company, Oak Ridge, Tennessee. Prior to injection of the labeled $BaCO_3$, 20 mg. of non-labeled $BaCO_3$ was injected into rabbits to test the possible toxicity of the barium ion. No effect was noted as evaluated by observation of the smooth muscle for spasms.

Immediately following injection of the C^{14} labeled carbonate, the animal was placed in a sealed chamber made out of an 11-gallon aquarium tank. Compressed air was forced through 3 traps and then into the sealed chamber. Starting from the compressed air line, the traps consisted of concentrated H_2SO_4, concentrated NaOH, and tap water, respectively. The air was sucked from the opposite end of the chamber by a water aspirator. Three concentrated NaOH traps were placed between the aspirator and the sealed chamber. A flow meter was connected into the system and the air flow was determined to be 500 ml./min. Also, a kerosene manometer was hooked up to the chamber and the input and output of air was regulated to atmospheric pressure.

The rabbit was kept in the chamber for 6 hours post injection. During this interval, air samples and samples from the three NaOH traps were taken periodically. There was no CO_2 buildup and all of the radioactive CO_2 expired by the rabbit was trapped in the first two NaOH traps.

At the end of the six-hour period, the rabbit was removed from the chamber and anaesthetized. A carotid artery and femoral vein were cannulated. Blood was drained from the carotid artery and physiological saline was run into the femoral vein at a rate of 20 ml./min. This was continued until the animal expired. The majority of the blood clotted shortly after collection in 500 ml. blood bottles. The mixture of clotted blood and blood diluted with saline was centrifuged and the dilute serum was fractionated by Cohn's et al. (1950) alcohol fractionation procedure (Method 10). Because of the dilution of the plasma with saline, slight adjustments of pH had to be made to attain the proper pH's according to Cohn *et al.* (1950).

The fractions obtained by the fractionation were albumins, γ-globulins, a crude fraction consisting primarily of β-globulins, and a crude fraction consisting primarily of α-globulins. The yields, specific activities, and percent of activity of the injected dose in the protein fractions from the blood of these two animals are reported in Table 1 of the appendix. All subsequent blood protein fractions were made in this manner. The α- and β-globulins referred to in the remainder of this text refer to these crude α- and β-globulin fractions.

3. Methods of Injection and Withdrawal of Rabbit Blood and Milk Samples

All injected materials were injected via the marginal ear vein. The amino acids and protein fractions were dissolved in a minimal amount of phosphate buffer (pH 7.2). Oxytocin was diluted to a concentration of 1.0 I.U./ml. of distilled water.

Blood samples were obtained by heart puncture with a 20 gage needle. An endeavor was made to withdraw the blood from the left ventricle for the sake of uniformity and consistency of composition. The blood sample was transferred immediately to conical plastic centrifuge tubes and allowed to clot at room temperature.

Milk samples were taken from the right and left inguinal glands. Just prior to milking, the animals were injected with one I.U. of oxytocin to stimulate milk ejection from the lobulo-alveolar system. This dose was found to be very effective.

The milk was withdrawn in the following way: A vacuum (-15 mm.) was established by means of a water aspirator. A 50 ml. flask was placed in the vacuum line to serve as a milk collecting vessel. A gum rubber tube was attached to the vessel. A beveled glass tube of the proper diameter to easily slip over the rabbit's nipple was inserted into the other end of the rubber tube. The beveled glass tube was placed on the nipple and the mammary gland vigorously massaged from the periphery toward the base of the nipple. The suction was interrupted every few seconds by removal of the beveled glass tube on the nipple. The procedure was continued until no more milk could be withdrawn from these two glands.

The inguinal glands were used because it was found by experience that they were the easiest pair to milk. It is felt that the use of oxytocin materially aided in removal of as much milk as possible from these glands. This was necessary to prevent mixing new milk formed with residual milk and thus causing errors in the determination of the radioactivity of the milk proteins. After milking the animals were returned to their litters. Animals were used only if they had a minimum of four in their litters.

This milking procedure was found to be very satisfactory after some experience had been obtained. It was found that extreme nervousness on the part of the rabbit inhibited the release of milk and perhaps milk production also. Therefore, a training period of a few days prior to the beginning of an experiment was helpful. Generally, the first attempt to milk a rabbit was difficult. After a few attempts, the animals became accustomed to the procedure. They would usually be very relaxed and no difficulty was experienced.

The time required to milk the two inguinal glands as completely as possible was in the order of 15 minutes. At the end of the milking, the blood samples were drawn. During the injection, milking, and bleeding, the animals were restrained on their backs on a small animal surgery board.

4. Fractionation of Rabbit Milk Proteins

The rabbit milk was centrifuged at 1°C for 30 minutes to remove the fat. The fat formed a semisolid plug on the surface of the milk. The skim milk was removed by inserting a pipette under the fat and removing the skim milk. This was diluted with an equal volume of distilled water.

Caseins were precipitated by adjusting the pH of the skim milk to pH 4.3 with small additions of 0.5 N HCl. It is of interest that the isoelectric point of bovine caseins is pH 4.7. The precipitated caseins and whey were separated by filtration through Whatman No. 42 filter paper.

The immune globulins were at first separated by the method of Smith (1948). During the adjustment of the pH of the whey to 6.0, a gelatinous precipitate appeared in the solution. The precipitation did not increase during the addition of ammonium sulfate to the proper concentration. Paper electrophoresis of this fraction showed that it had the same mobility as the immune globulin fraction of the original whey when it was subjected to electrophoresis at the same time. Another sample of acid whey was adjusted to pH 6.0. The same precipitate was obtained. No ammonium sulfate was added. The precipitate was filtered off and subjected to paper electrophoresis. This precipitate from pH adjustment only, appeared to be the same as the one to which ammonium sulfate had been added. Therefore, in rabbit milk it appears that the immune globulin fraction can be obtained by pH adjustment only. This protein fraction was homogeneous by the criterion of paper electrophoresis.

Therefore, this method was used to isolate the protein fraction referred to as rabbit milk immune globulins in the Results.

The α-lactalbumin fraction was isolated by the method of Gordon and Semmett (1953). β-lactoglobulin was isolated by the method of Larson and Jenness (1955).

The "blood" serum albumin fraction was first noticed on electrophoresis patterns of rabbit whey. The method of Polis _et al._ (1950) was used to obtain the "blood" serum albumin fraction from the rabbit whey.

5. Dialysis of Rabbit Blood and Milk Protein Fractions

When the various fractions were obtained, they were transferred to dialysis sacs prepared from Visking tubing. The fractions were dialized against frequent changes of distilled water until free from salts, alcohol, and other reagents used in the fractionation procedures. This was usually complete in about 5 days. It was also felt that this procedure might help to remove any free radioactive amino acid which might have adhered to the protein during the fractionation procedures.

6. Lyophillization of Protein Fractions

When dialysis was complete, the fractions were transferred to wide-mouth rat water bottles. The solutions were frozen in a shell of uniform thickness around the inside surface of the bottle. This was accomplished by inserting the bottles into a bath of alcohol and dry ice. As the bottles cooled, they were rotated to facilitate the shelling of the ice on to the surface of the bottle. The bottles were never filled to more than half their capacity.

The bottles were attached to the lyophillizer one at a time after the refrigeration bath was cooled to -50°C and high vacuum had been established. A 5-minute interval was used between the placing of samples on the lyophillizer. This was done to prevent overloading of the apparatus and loss of vacuum and thus melting of the samples. After the ice had been sublimed and the samples were dried, they were removed and stored for electrophoresis and counting.

7. Paper Electrophoresis of Rabbit Serum, Whey, Blood, and Whey Protein Fractions

Paper electrophoretic patterns were run in Beckman/Spinco paper electrophoresis cells. The procedure used for developing and staining these blood serum and blood protein fractions is outlined in the manual for the operation of the Beckman/Spinco electrophoresis cell.

Rabbit whey and whey protein fractions were first run according to the procedure of Leviton (1957). This procedure was not too satisfactory because the large β-lactoglobulin fraction with highest mobility in the buffer system used was absorbed on the paper strip along the entire length of the pattern. This absorption masked the peaks of the other whey protein fractions.

Due to this difficulty, a method was developed to overcome it. The paper strips were soaked in a 5% glycerol solution containing 0.5% gelatin for 10 minutes. The glycerol was used to prevent the paper strips from becoming stiff. Next, the absorbed gelatin was fixed on the paper strips by dipping them in 5% formaldehyde for a few minutes. Then the paper strips were washed in tap water and distilled water to remove any excess gelatin and formaldehyde. The strips were dried at 125°C to completely denature the absorbed gelatin.

When these gelatin treated strips were used for the paper electrophoresis of whey proteins, very satisfactory patterns were obtained. This method has been used for all electrophoresis of whey samples and whey proteins. The gelatin absorbed on the paper strips and denatured by formaldehyde and heat did not stain with bromophenyl blue. It was not detected when the patterns obtained were run through the analytrol.

Paper electrophoretic patterns were run on all serum samples and protein fractions from these samples. This was also done for whey and whey protein fractions. These paper electrophoretic patterns were utilized to check the identity and homogeneity of the isolated fractions by comparison to patterns run on the original serum or whey. Figure 2 in the appendix is a photograph of a set of paper electrophoresis patterns from an original serum sample, and the fractions isolated from it. Figure 3 in the appendix is a photograph of a set of paper electrophoresis patterns from whey and the fractions isolated from it.

During preliminary investigations, an attempt was made to separate the various protein fractions of rabbit serum and whey by the Continuous Flow Curtain Electrophoresis Apparatus. Some separation was achieved but the yields were low. Also, the separation into homogenous fractions was not very good. This was especially true in the case of the blood serum.

8. Procedures for Estimation and Isolation of Free Leucine and Glutamic Acid in Rabbit Blood Serum

An equal volume of 20% trichloroacetic acid was added to a small volume of rabbit serum to precipitate the serum proteins. The precipitate was filtered and washed with 3 small portions of distilled water, using Whatman's No. 42 filter paper. The filtrate was extracted 5 times with ether to remove the excess trichloroacetic acid. This filtrate was then subjected to column chromatography by the method of Moore *et al.* (1958). The filtrate had to be added in small portions and eluted from the columns a number of times due to the low capacity of the columns. The amount of leucine or glutamic acid in the eluent was determined colorimetrically.

by development of the color in an aliquot with ninhydrin (Moore et al. 1958). The eluent was pipetted into a scintillation bottle and evaporated to dryness in a vacuum oven at 70°C. Then it was stored for counting.

The amount of free leucine or glutamic acid in the serum was calculated from the following equation:

$$\frac{\text{mg. of amino acid/ml. x ml. of eluent x 100}}{\text{ml. of original serum sample}} = \text{mg. \% in serum}$$

The mg. % of free leucine in serum samples from animal X-35 (Experiment I) is presented in Table 3 in the appendix.

The amount of the free blood amino acids in the scintillation bottles was calculated from the following equation:

mg./ml. of eluent x ml. of eluent in the bottle =
mg. of free blood amino acid counted

It should be remembered that the data reported in the tables in the Results under the heading free blood leucine or glutamic acid counted were determined in the way reported in this section. It is not a weight determined gravimetrically. It is recognized that there are inherent errors in a procedure such as this. However, Moore et al. (1958) report a recovery of 99% on synthetic mixtures of amino acids. It is felt that this procedure is more accurate than attempting to weigh 1 to 2 milligrams of material.

9. Procedure for the Estimation of the Percent Leucine and Glutamic Acid in Blood and Milk Protein Fractions

Samples weighing from 40 to 50 mg., when this much material was available, were weighed into small flasks. Twenty milliliters of 20% HCl were added and the samples autoclaved for a minimum of 10 hours at 15 lbs. pressure to hydrolyze the proteins. Ten samples of each blood and milk protein were hydrolyzed in this way. The hydrolysates were neutralized. Aliquots of these hydrolysates were subjected to column chromatography (Moore et al. 1958). The leucine and glutamic acid were determined in the eluents by the method of Moore et al. (1958). The average values for leucine and glutamic acid from these fractions is reported in Table 4 in the appendix.

10. Procedure for the Isolation of Glutamic Acid from Serum and Milk Protein Fractions for Estimation of the Specific Activity of Glutamic Acid

Glutamic acid can be rapidly metabolized to arginine, proline and hydroxyproline. These amino acids are then available for synthesis into proteins. Also, a major portion of glutamic acid in proteins can be in the form of glutamine. Therefore, it was necessary to isolate the glutamic acid in the protein fractions. If the radioactivity in the whole protein fractions had been counted, it would not have been possible to compare the activities with those found for free blood glutamic acid.

Samples of the protein fractions were weighed. A standard amount of 10 mg. of pure glutamic acid was added to each sample. This mixture was hydrolyzed as described in the previous section. The insoluble chloride of glutamic acid was precipitated by cooling the hydrolysate to 0°C according to the procedure of Fischer (1901). The material was dissolved by addition of 0.5 N NaOH and reprecipitated by the addition of 6 N HCl and cooling to 0°C. This material was washed with .01 N HCl and then with acidified alcohol and dried. Paper chromatographs were prepared as described earlier and only one spot was evident upon development with ninhydrin. The R_f value was that of glutamic acid.

The dried chloride of glutamic acid was weighed into a scintillation bottle for counting. The weight was corrected to the weight of free glutamic acid by the following equation:

$$\text{wt. of free G.A.}[1] = \frac{(\text{M.W.}[2] \text{ of G.A.}) \times (\text{wt. of the chloride of G.A. isolated}}{(\text{M.W. of the chloride of G.A.})}$$

The theoretical amount of glutamic acid in the hydrolysate was calculated from the following equation:

$$\text{Theoretical amount of G.A.} = (\%\ \text{G.A. in the protein fraction} \times \text{wt. of the fraction hydrolyzed} \times 10^{2}) + 10\ \text{mg.}$$

The amount of glutamic acid weighed into the scintillation bottle derived from the hydrolyzed protein fraction was calculated by the following equation:

$$\text{mg. G.A. counted} = \frac{(\text{wt. G.A. in the scintillation bottle}) \times (\text{wt. G.A. in the hydrolyzed protein})}{\text{theoretical wt. of G.A. in the hydrolysate}}$$

The weights of glutamic acid derived from the various protein fractions reported in Tables 7 and 10 in the Results were determined in this manner. It is obvious that there are many possible sources of error in this procedure. However, it was felt that this procedure would be the most efficient and would provide reliable data.

When glutamine is subjected to acids it is converted to free glutamic acid. Therefore, the values reported as free glutamic acid in blood and protein fractions is actually a combination of glutamic acid and glutamine. This is permissible since glutamine is derived from glutamic acid.

11. Procedure for the Estimation of Radioactive Leucine in the Blood and Milk Protein Fractions

Leucine is metabolized to acetoacetic acid and acetyl-CoA. The

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[1] G.A. = glutamic acid.

[2] M.W. = molecular weight.

number 2 carbon of the leucine-2-C^{14} becomes the carboxyl carbon in the acetyl portion of acetyl-CoA. Thus, it is evident that this isotopic carbon atom can become incorporated into a large variety of compounds via the Krebs cycle and the pathways for fat synthesis. However, the dilution of this isotopic carbon atom by other metabolic pathways leading to acetyl-CoA is considerable. Also, adequate supplies of amino acids were available from the diet. Because of these considerations, it was not deemed necessary to isolate leucine from the protein fractions. It was felt that the error introduced by radioactivity contributed to the proteins due to the metabolism of leucine and subsequent incorporation into non-essential amino acids would not be any greater than the errors introduced in an isolation procedure for leucine from protein fractions.

Forty to fifty milligrams, or as much as was available, of the protein fractions were weighed into scintillation bottles and stored for counting. The weight of leucine in these fractions was calculated from the average percent of leucine in these fractions as determined earlier and reported in Table 4 of the appendix. Thus, the mgs. of leucine counted reported in Tables 1 and 4 in the results is a calculated value based on the percent of leucine in the weighed protein fractions.

12. Determination of the Specific Activity of C^{14} Protein Fractions Produced by the Injection C^{14} Labeled $BaCO_3$

These fractions were simply weighed into the scintillation bottles. Samples of 40 to 50 mg. were weighed when this much material was available. The C^{14} would be randomly distributed in the non-essential amino acids of these proteins. Therefore, the activity in these samples was calculated as µc./mg. of protein.

13. Sample Preparation, Counting Procedure, and the Calculation of Specific Activity

All samples were counted in the Tricarb β-Liquid Scintillation Spectrometer manufactured by the Packard Instrument Company, Inc., LaGrange, Illinois. This instrument is ideally suited to the counting of carbon-14 compounds of low specific activity because of its high sensitivity to low energy β-particles and its very high counting efficiency.

The samples were weighed into the scintillation bottles as described previously. Two milliliters of hydroxide of hyamine in methyl alcohol were added to the dry proteins or amino acids to take them into solution. The chemical name of hydroxide of hyamine is p-(diisobutyl-cresoxy-ethoxyethyl) dimethylbenzyl-ammonium hydroxide. The mixtures were warmed to 50°C in a water bath until the solutions were perfectly clear.

Next, 5 ml. of the scintillation fluid was added to the dissolved protein hyamine mixture in the bottles. The scintillation fluid is composed of 6 gm. PPO plus 100 mg. POPOP dissolved in 1 lt. of A.C.S. grade Toluene. PPO is the trade name for (2,5-diphenyloxazole) and POPOP is the trade name for 1,4-di[2-(5 phenyloxazole)] benzene. This scintillation fluid serves the function of transforming the energy of the β-particle emitted from the unstable C-14 atom into light energy which is, in turn, transformed into electrical energy by the light sensitive photocells surrounding the sample bottle.

The liquid scintillation system has some disadvantages. The primary problem is that hyamine and other compounds have a quenching effect on the scintillation fluid. This means that some of the disintegrations will not be recorded because the fluorescence produced is quenched before it can be recorded by the photocells and thus counted by the instrument.

The quenching of hyamine was determined by adding a radioactive standard of 2 ml. of hyamine plus 5 ml. of the scintillation fluid.

When enough material was available, samples were prepared in triplicate. Caseins, β-lactoglobulins, albumins, and in some cases γ-globulins and immune globulins were prepared in triplicate. Also, samples of non-radioactive glutamic acid and sodium citrate were dissolved in hyamine and scintillation fluid added. These samples were spiked with a standard of benzoic acid-C^{14} made up in toluene. This standard was prepared to have a theoretical count of 1046 disintegrations per ml. per minute. Also, bottles were prepared containing hyamine and scintillation fluid only for determining background.

The samples were placed in the automatic sample changer in the freezer for a minimum of 4 hours. This time is necessary to cool the samples and to allow for any residual fluorescence due to sunlight to dissipate. The instrument was set up so that each sample was counted for a period of 5 minutes and automatically recorded on a paper tape.

The standards plus hyamine counted at an efficiency of 50%. The counts of the duplicate protein samples always counted within 2% of each other. These were averaged and compared to the count of the third sample plus the spike of 1 ml. of the radioactive standard (1046 DPM). The spiked sample should have had a count of 523 CPM higher than the average count of its two duplicates since the counting efficiency was 50%. The average difference between the averages of the two protein samples and the spiked sample for all of the samples tested in this way (a total of 50 fractions) was 450.7 ± 10.6[1] higher for the spiked

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[1] The average with standard error.

sample. The quenching due to the protein fractions may be calculated in the following way:

$$\frac{523.0 - 450.7}{523} \times 100 = 13.8\%$$

This means that 13.8% of disintegrations were not counted due to quenching of the fluorescence due to the proteins. No quenching was observed in the glutamic acid or citrate samples. The background averaged 11.0 ± 1.5[1] CPM[2].

Since the counting efficiency was 50% and the quenching due to proteins was 13.8% and background was 11.0 CPM, the following correction factor was applied to convert the raw count of the instrument for the 5-minute period to DPM[3] in the case of proteins:

$$\text{DPM} = \frac{(\text{raw 5 min. count}) \times 100 \times 100}{86.2 \times 50 \times 5}$$

The amino acids were corrected to DPM by the following equation:

$$\text{DPM} = \frac{(\text{raw 5 min. count}) \times 100}{50 \times 5}$$

The term $\frac{100}{86.2}$ is dropped from the second equation since no correction is necessary for quenching in the case of the amino acids.

The tables in the Results report the activity in terms of µc./mm.[4] for the amino acids. One µc. of an unstable isotope is defined as that amount which disintegrates at a rate of 222×10^4 DPM. Therefore, the µc./mm. are calculated by the following formula:

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[1] The average background with standard error.

[2] CPM = counts per minute.

[3] DPM = disintegrations per minute.

[4] µc./mm. = microcuries per millimole.

$$\mu c./mm. = \frac{\text{DPM x wt. of amino acid counted in mg.}}{222 \times 10^4 \text{ x millimolecular wt. in mg.}}$$

The millimolecular weights of leucine and glutamic acid in mg. are 131 and 147, respectively.

The activities of the protein fractions in the tables in the Results are reported as μc./mg. of protein counted. These values may be calculated in the following manner:

$$\mu c./mg. \text{ of protein counted} = \frac{\text{DPM}}{222 \times 10^4 \text{ x mg. of protein counted}}$$

14. Data on Urine and a Summary of Animals Used in Experiments I Through X

Table 5 in the appendix presents data on the activity in urine collected on animal X-35 injected with 45 μc. of leucine-C^{14}. The animals frequently urinated immediately following the injection of oxytocin. Small samples of the urine were collected when this occurred. The urine was placed in a scintillation bottle, dried and stored for counting. These counts are reported as CPM/ml. of urine. These samples did not yield perfectly clear solutions when treated with hyamine. It is difficult to interpret how much quenching effect this caused. Therefore, the reliability of these data are questionable.

Table 2 in the appendix presents a summary of the animals used in the studies of rabbit milk proteins. It describes the animals, materials injected into these animals, and amounts of milk and blood withdrawn.

B. Methods Used for In Vitro Studies of Guinea Pig Mammary Tissue

1. Cleaning of Glassware

The proper cleaning of glassware is one of the most important techniques in tissue culture. Two problems are encountered: (1) toxicity due to improper rinsing and (2) bacterial contamination due to incomplete cleaning. Micro-Solv was used as the cleaning agent for the glassware. This is a special cleaning agent available from Microbiological Associates, Inc. It can be thoroughly rinsed away with water and has very low toxicity properties. It has a pH of 7.0.

The glassware is scrubbed vigorously in very hot micro-solv solution until distilled water will run from the glass in a sheet, leaving a thin unbroken film of water on the surface of the glass. Then it is rinsed 5 times with hot tap water, 5 times with regular distilled water, and 15 times with triple glass distilled water. The glassware is then air dried, wrapped in paper towels, and sterilized by dry sterilization at 150°C for one hour or longer.

2. Preparation of Media

Parker's synthetic "199" was used as the basic medium. This medium was originally developed by Morgan *et al.* (1950). This medium contains all the essentials known for the nutrition of cells *in vitro*. It does not contain any hormones. This medium is available in a concentrated form from Microbiological Associates, Inc. The medium contains 10 times the concentration for use and must therefore be diluted. This is accomplished by placing 41 ml. of triple distilled water in a prescription bottle and autoclaving it for 2 hours at 15 lbs. pressure. After cooling the sterile water, 5 ml. of the stock sterile "199" is removed from its container by means of a sterile needle and syringe and transferred aseptically to the prescription bottle. The pH of this solution is acid. The "199" contains methyl red as an indicator. The pH is adjusted to 7.2 by the aseptic dropwise addition of sterile 1.4% $NaHCO_3$. The pH is judged by comparison of the color to methyl red solutions of known pH. The medium is then gassed with 95% O_2 and 5% CO_2 by bubbling the gas through the medium by means of sterile glass tube drawn out to a very fine capillary.

Sterile stock solutions of hydrocortisone (800 µg./ml.), prolactin (12,000 µg./ml.), insulin (7,000 µg./ml.), and penicillin (2,500 I.U./ml.) were prepared in advance. One ml. of each of these stock solutions was then added to the medium aseptically. Five milliliters of sterile rabbit serum was then added. This procedure yields 50 ml. of medium II reported in Table 22 of the Results. Medium I was prepared in the same manner, except 42.5 ml. of water is sterilized and .5 ml. of the hydrocortisone, prolactin, and insulin stock solutions are added.

Mediums containing radioactive amino acids or protein were prepared in the same manner. However, aseptic technique was not required since the radioactive materials are not sterile. The radioactive materials are added last in the desired concentrations. The medium is then sterilized by passage through a bacteriological filter and transferred aseptically to a sterile prescription bottle.

3. Tissue Culture Method

The method of culture used in the tissue culture experiments was the Chen (1954) modification of Fell's (1929) organ culture method, which has been further modified by Shaffer (1956). It was found that the medium tended to evaporate when cultured by this method. This preparation consists of a watch glass supported by moist cotton in a

Petri dish. The medium is placed in the watch glass and a treated cellulose acetate raft holding the explants is floated on top of the medium.

It was found that the medium evaporated during the culture period. Therefore, the moist cotton was replaced by very wet filter paper pulp. This procedure all but eliminated the media evaporation problem. The completed setups were placed in coffee cans and autoclaved for 2 hours at 15 lbs. pressure to insure sterilization. Just prior to sacrifice of the animal, 1 ml. of the desired media was placed in each watch glass.

4. Removal of Mammary Tissue and Preparation of Explants

Non-gravid guinea pigs that had not been suckled for 30 days were used to provide non-secretory mammary tissue for culture. Four to six day postpartum guinea pigs were used to provide secretory mammary tissue for culture.

The animals were sacrificed by a blow on the head. The inguinal areas were scrubbed with Roccal solution (800 PPM.) to sterilize the skin. An incision was made in the skin and the mammary tissue removed and placed in sterile basic salt solution. An area of the gland was selected which appeared to have good lobulo-alveolar development. This was cut into explants approximately 1 mm. in diameter and thickness. The remainder of the mammary tissue was fixed in Bouin's fluid for 4 hours to serve as a histological control. Three explants were transferred aseptically to each raft. The rafts were then floated on the media in the watch glass. The cultures were transferred to an incubator at 37°C. The time required to sacrifice the animals, prepare the explants and get them into culture was approximately 30 minutes.

5. The Culture Period

The cultures were checked daily for pH changes in the media. At pH 7.2 the medium is a deep pink; reddish orange at pH 6.9; and yellow at lower pH's. When the medium changed to a reddish orange color, it was removed by suction into a sterile glass tube drawn out to a fine capillary. Fresh media was added, the raft refloated, and the culture continued. The medium was normally changed every other day.

Cultures were normally terminated after 5 days. Cultures terminated prior to 5 days did not show a maximal response to the hormones. Cultures maintained longer than 5 days showed progressively greater degeneration.

6. Preparation of Stained Histological Sections of Explants and Control Tissues

The explants were transferred to Bouin's fluid for 1 hour for fixation. The larger control tissue was fixed for 4 hours in Bouin's fluid. The tissues were transferred to 70% alcohol. The control tissue was kept in the 70% alcohol until the explants were ready to be transferred into 70% alcohol. The tissues were dehydrated by running them through the alcohol series and into zylol. They were infiltrated with low melting point paraffins and subsequently changed to higher melting point paraffins. They were finally embedded in 54-56° melting point paraffin. Care was taken to embed the three explants from the same raft on the same plane in a block. In this way a single section on a slide would contain tissue from all three explants.

The blocks were shaped and cooled prior to cutting sections. Sections were cut at 6 μ and fixed on microscope slides. They were stained with iron hematoxylin and eosin and coverslipped in the usual manner.

7. Preparation of Autoradiographs

Autoradiographs were prepared by a modification of the method of Gross et al. (1951). Four slides of each stained tissue cultured in a radioactive medium were prepared in the usual manner but coated with celloidin instead of coverslipping. The slides were coated with a thin layer of Eastman Kodak type NTB emulsion in the dark, placed in a light proof slide box in a horizontal position and stored in the refrigerator. Periodically, these slides were developed to ascertain the extent of exposure of the emulsion due to the radioactivity. The time was usually of the order of 2 to 6 weeks.

The emulsion was developed for 15 minutes in dektal (Eastman Kodak) at 15°C, then fixed with acid fix for 30 minutes at 15°C. The slides were left in cold running tap water overnight, then dehydrated by running through the alcohol dehydration series. After dehydration, they were coverslipped in the usual way. Longer periods of time for treatment with dektal, acid fix, and washing were found necessary in order to completely clear the emulsion.

This method permits evaluation of the histological condition of the tissue and examination of the autoradiograph on the same preparation. Thus, the concentration of radioactivity can be associated with specific areas of the tissue. The tissue and emulsion are on different planes. Thus, it is possible to examine tissue microscopically, then without changing the field, refocus on the emulsion and study it. The development of the emulsion affects the stain by reducing contrast, but not sufficiently so that the tissues cannot be evaluated.

8. Evaluation of the Morphological Condition of the Explants

The alveoli of each explant in 5 fields at high power selected at random were counted. The same procedure was carried out on the control tissues. Thus, a comparison could be made and a very rough estimation of the percent of the lobulo-alveolar system maintained *in vitro* could be calculated. This is reported in Table 24 in the Results.

The secretory tissues could not be evaluated by the above manner because of the differences in sizes of the secretory alveoli in the controls and explants. Many of the secretory explants contained much larger alveoli than the controls. Therefore, in these explants it was necessary to count the number of non-secretory, secretory, and degenerate alveoli in the 5 fields selected at random. The percent of the lobulo-alveolar system maintained and the percent of lobulo-alveolar system which were secretory are reported in Table 25. The results are calculated in the following manner:

$$\%\text{ of LA}^{1}\text{ maintained} = \frac{\text{LA maintained} \times 100}{\text{maintained LA} + \text{secretory LA} + \text{degenerate LA}}$$

$$\%\text{ of LA which was secretory} = \frac{\text{secretory LA} \times 100}{\text{maintained LA} + \text{secretory LA} + \text{degenerate LA}}$$

It is recognized that this method of evaluation of maintenance and secretion is highly subjective. At best, it is only a very general estimation of the actual condition. It is possible that the average values reported in Tables 24 and 25 in the Results could have an error of 25% or perhaps even more. However, this measure does provide some information on the histological condition of the explants.

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[1] LA = lobulo-alveolar tissue.

RESULTS AND DISCUSSION

I. Studies on the Precursors of Rabbit Milk Proteins

A. Experiment 1. The Incorporation of Leucine-2-C^{14} into Serum and Milk Proteins by the Lactating Rabbit

1. Decrease in the Specific Activity of the Extracellular Free Leucine Pool

The specific activities of leucine from the various blood and milk protein fractions from X-35 are presented in Table 1. The specific activity of the free leucine of the blood decreases at an extremely rapid rate during the first 10 minutes post injection. It is evident from an inspection of the data for the specific activities of free leucine in the blood presented in Table 1 and Figure 1 that a model of the biological system operating for the removal of free leucine must be postulated. Rabbit No. X-35 weighed 5,000 gm. If it is assumed that 20% of the body weight is interstitial fluid or extracellular water, then it may be calculated that this extracellular pool is 1,000 ml. If 7% of the body weight is blood and 50% of the blood is plasma, then it may be calculated that the animal had 175 ml. of plasma. The data in Table 3 in the appendix give an average value of 2.6 mg.% of free leucine in the serum. If these values are assumed to be approximately correct, the following calculations can be made:

1,000 x .026 = 26 mg. free leucine in HOH_{ex}[1]

175 x .026 = 4.55 mg. free leucine in plasma.

A total of 45 µc. in 11.5 mg. of leucine was injected into Rabbit No. X-35.

45 µc. in 11.5 + 26 = 37.5 mg. = 37.5/131 = 0.2863 mm. of free leucine in the extracellular pool at 0 time.

45/.2863 = 157.2 µc./mm. in HOH_{ex} at 0 time.

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[1] HOH_{ex} = extracellular water.

TABLE 1. SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-35, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 μC. OF LEUCINE-2-C^{14}

hrs. post inj.	DPM[1]	mg. leucine counted	μc./mm. x 10^2
		Free Blood Leucine	
0.16	12,110	0.19	370.00
2	8,100	0.21	230.00
6	4,660	0.16	170.00
12	2,827	0.17	98.00
24	500	0.18	28.00
36	148	0.21	7.00
48	70	0.20	2.10
72	7[2]	0.25	0.18
		Leucine From Casein	
2	105,700	4.21	148.00
6	102,200	4.99	122.00
12	51,600	3.65	83.50
24	32,450	3.87	49.50
36	13,260	4.80	16.30
48	2,538	4.54	3.30
72	322	5.00	0.38
		Leucine From Milk β-Lactoglobulin	
2	89,400	2.1	250.00
6	161,700	4.7	200.00
12	97,800	3.9	150.00
24	27,000	3.2	50.00
36	8,850	4.5	12.00
48	2,890	5.0	3.40
72	975	6.3	0.58
		Leucine From Milk α-Lactalbumin	
2	68,900	3.3	120.00
6	81,350	4.0	120.00
12	42,150	2.7	92.00
24	27,300	3.5	46.00
36	5,110	2.2	14.00
48	3,640	3.8	5.60
72	761	4.4	1.00

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[1] DPM = disintegrations per minute. The calculation of DPM and μc./mm. explained in the Methods section.

[2] The count of this sample was not significantly different from background.

TABLE 1. (CONT.) SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-35, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 μC. OF LEUCINE-2-C^{14}

hrs. post inj.	DPM	mg. leucine counted	μc./mm. x 10^2
		Leucine From Milk Immune Globulins	
2	11,570	2.41	28.30
6	31,400	3.15	58.90
12	26,600	2.96	53.00
24	14,310	2.19	38.60
36	9,130	1.87	28.80
48	8,610	2.54	20.00
72	5,450	3.28	9.80
		Leucine From Blood γ-Globulins	
2	15,650	2.99	30.90
6	25,490	2.45	61.40
12	30,350	3.06	58.50
24	13,340	1.97	40.00
36	11,400	2.28	29.50
48	13,300	3.10	25.30
72	6,820	3.44	11.70
		Leucine From "Blood" Serum Albumin From Milk	
2	705	0.21	20.00
6	1,110	0.26	25.00
12	1,150	0.30	23.00
24	774	0.28	16.00
36	581	0.25	14.00
48	423	0.29	8.60
72	347	0.32	6.40
		Leucine From Blood Albumin	
2	14,520	3.90	22.00
6	19,650	4.22	27.50
12	13,220	3.56	21.90
24	9,390	3.13	17.70
36	11,800	4.57	14.90
48	6,455	4.28	8.90
72	6,340	4.98	7.50

TABLE 1. (CONT.) SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-35, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 μC. OF LEUCINE-2-C^{14}

hrs. post inj.	DPM	mg. leucine counted	μc./mm. x 10^2
		Leucine From Blood α-Globulins	
2	1,779	1.98	5.31
6	7,880	2.46	18.90
12	6,410	2.80	13.50
24	2,655	1.59	9.85
36	3,620	2.54	8.41
48	2,818	3.02	5.50
72	2,715	3.47	4.62
		Leucine From Blood β-Globulins	
2	1,445	1.15	7.42
6	7,960	1.56	30.10
12	5,680	1.29	26.00
24	5,510	1.87	17.40
36	2,340	1.34	10.30
48	2,495	1.75	8.40
72	1,892	2.23	5.00

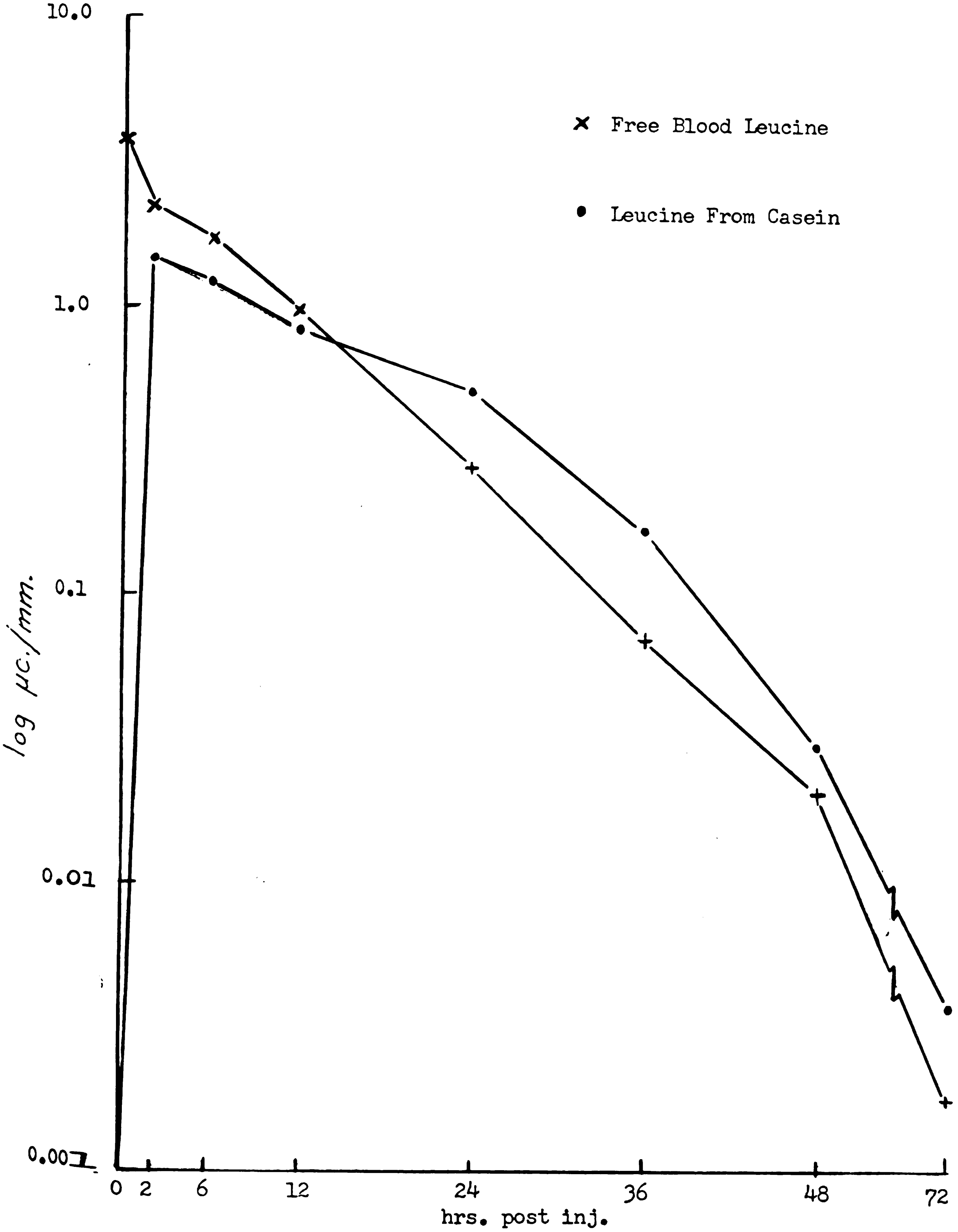

FIGURE 1. THE LOG OF THE SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM CASEIN ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

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It may be seen from inspection of the free blood leucine curve presented in Figure 1 that the rate constant (k) for the first 10 minutes (0.16 hrs. post injection) is much greater than the rate constant after 10 minutes. It should be pointed out that the first plot for free blood leucine plotted on the graph (Figure 1) represents the specific activity at 10 minutes post injection. The theoretical value at zero time is not represented on this curve. Inspection of the data presented in Table 1 shows that the specific activity of free blood leucine is 3.7 µc./mm. at 10 minutes (0.16 hrs. post injection). The first process from 0 to 10 minutes may be described by the following equations:

$$\ln \frac{3.7}{157.2} = k(10\ \text{min.}) = \ln 0.0235 = -3.75$$

$$k = -0.375\ \text{min.}^{-1}$$

$$t_{\frac{1}{2}} = \frac{.693}{.375} = 1.85\ \text{min.}$$

This means that 37.5% of the leucine was being removed from the extracellular pool each minute or that half of the leucine in this pool was exchanged with non-labeled leucine each 1.85 minutes. These data may also be expressed in the following manner:

(1) 3.7 x 0.2863 mm. of free leucine in HOH_{ex} = 1.06 µc. out of the 45.0 µc. injected were still in HOH_{ex} after 10 minutes.

(2) 3.7 x 0.0347 mm. of free leucine in plasma = 0.1284 µc. out of the 45.0 µc. injected were still in the plasma pool after 10 minutes.

This is based on the assumption that the size of the plasma and HOH_{ex} pools remain constant.

Based on the calculations above, it seems reasonable that during the first few minutes this system is one in which there is a rapid uptake of leucine with little or no return of the labeled leucine into the HOH_{ex} pool. It should be pointed out that the extracellular pool is assumed to be composed of the interstitial fluid and plasma. A system such as this can be described by the model for the biological system presented in Figure 2. There are several possible explanations for this system during the first few minutes. Possibilities are that the excess leucine is bound by the reticulo-endothelial system (RES); that some fraction of the plasma protein physically binds this excess leucine; and that this excess is rapidly incorporated into the intracellular leucine pool.

It is recognized that the model presented in Figure 2 is a gross oversimplification of the true system. The assumption is made in the construction of this model that there is an almost instantaneous equilibration of leucine between the extracellular pool and the plasma pool, and that for practical purposes they may be treated as one pool. The rate constant ($k = -0.375$ min.$^{-1}$), the simple exponential equation, and the $t_{\frac{1}{2}}$ value (1.85 min.) are undoubtedly made up of at least three individual rates and equations. It appears that this process is so complex that the limited data available are not sufficient to permit a complete mathematical treatment of this process. However, the data presented in Figure 2 might be a reasonably close approximation of the over-all process.

Inspection of the data presented in Table 1 and Figure 1 reveal that from 2 to 48 hours the curve for the decrease in the specific activity of free blood leucine is almost a straight line. The slight variation in the individual points plotted might be due to experimental

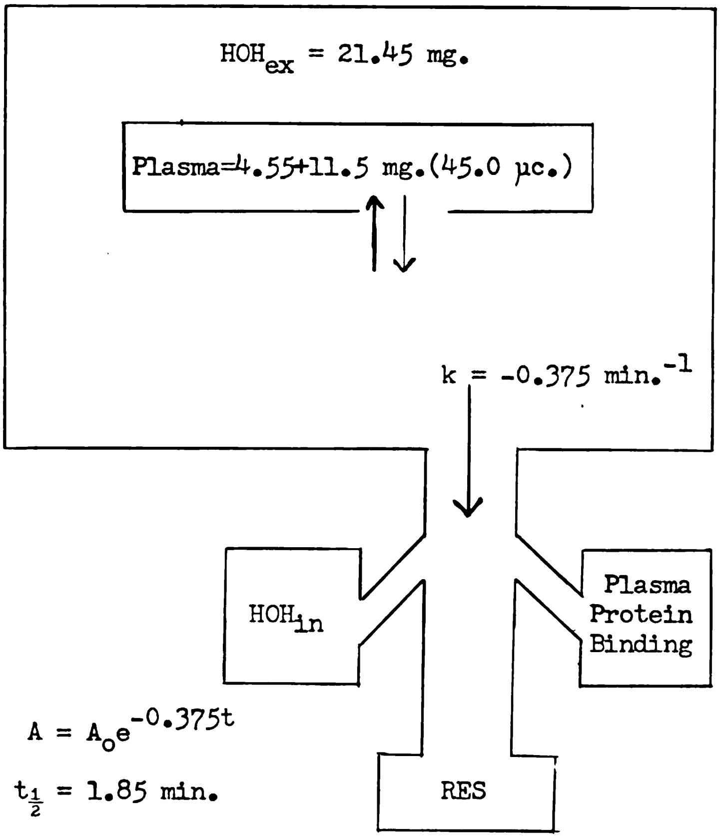

FIGURE 2. MODEL FOR THE REMOVAL OF DL-LEUCINE-2-C^{14} FROM THE EXTRACELLULAR POOL TO THE LEUCINE POOL OF THE RETICULO-ENDOTHELIAL SYSTEM (RES), THE INTRACELLULAR POOL (HOH_{in}) AND PLASMA PROTEIN BINDING.

error. The rate constant for this portion of the curve from 2 to 48 hours and the $t_{\frac{1}{2}}$ value can be calculated in the following way:

$$\ln \frac{0.021}{2.3} = k(46 \text{ hrs.}) = \ln 0.00914 = -4.68$$

$$2760\ k = -4.68$$

$$k = -0.00169 \text{ min.}^{-1}$$

$$t_{\frac{1}{2}} = \frac{.693}{.00169} = 409.5 \text{ min.}$$

These calculations are interpreted to mean that from 2 hours post injection, the net loss in the specific activity of the leucine was 0.169% of the total in the extracellular pool each minute or that the specific activity decreased 50% every 409.5 minutes.

It has been stated earlier in this discussion that the extracellular pool of leucine was in the order of 26 mg. prior to the injection of the labeled leucine. If the extremely rapid rate of disappearance of the labeled leucine during the first 10 minutes is considered and also that the 10-minute value for free blood leucine is 2.9 mg.% (Table 3, appendix), then it seems reasonable to assume that the extracellular leucine pool has returned to its normal size by 2 hours post injection. The rabbit in heavy lactation consumes a tremendous quantity of feed. This has varied from 300 to 400 gm. per 24 hours depending on the individual animal. These animals were fed a standard diet of rabbit pellets which contained a minimum of 18% protein. The average percent leucine in animal feeds is 2%, Block and Weiss (1956b). Assuming that these data are correct, then it is possible to calculate that the rabbit consumed an average of 1,440 mg. of leucine per day and absorbed 1 mg./min. from the gut.

If it is assumed that the rate constant for the first 10 minutes post injection of the leucine from the extracellular pool remains consta

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constant ($k = -0.375\ \text{min.}^{-1}$, $t_{\frac{1}{2}} = 1.85$), then this pool is turned over at a rate of 14.05 mg./min. as determined by the following equation:

$$\frac{26}{1.85} = 14.05\ \text{mg./min.}$$

If the same reasoning is applied to this pool after 2 hours, the following value is obtained:

$$\frac{26}{409.5} = 0.063\ \text{mg./min.}$$

Since there is such a drastic change in the decrease in the specific activity of the leucine in this extracellular pool, it must mean that during the latter period of the curve there is a return of the label to this extracellular pool.

If the size of the extracellular leucine pool remains constant (26 mg.) and the pool is turned over at a rate of 14.05 mg./min. as calculated from the $t_{\frac{1}{2}}$ of the first 10 minutes of the curve and also, that the pool is diluted with 1 mg./min. of leucine from the gut, then a return of approximately 13 mg./min. of labeled leucine to this extracellular pool must take place. A proposed model for this system is presented in Figure 3.

The over-all equation for this process described graphically in Figure 3 is presented along with the model. It is certainly true that the actual equation for this process is highly complex and is composed of at least 3 and in all probabilities more than 3 components. It is so complex and the data so limited that it is impossible to derive the true equation for this process.

Careful consideration of these data and mathematics lead toward the idea that perhaps only a small portion of the injected dose (3.7 µc.) behaved in the blood as free leucine and that this was the actual

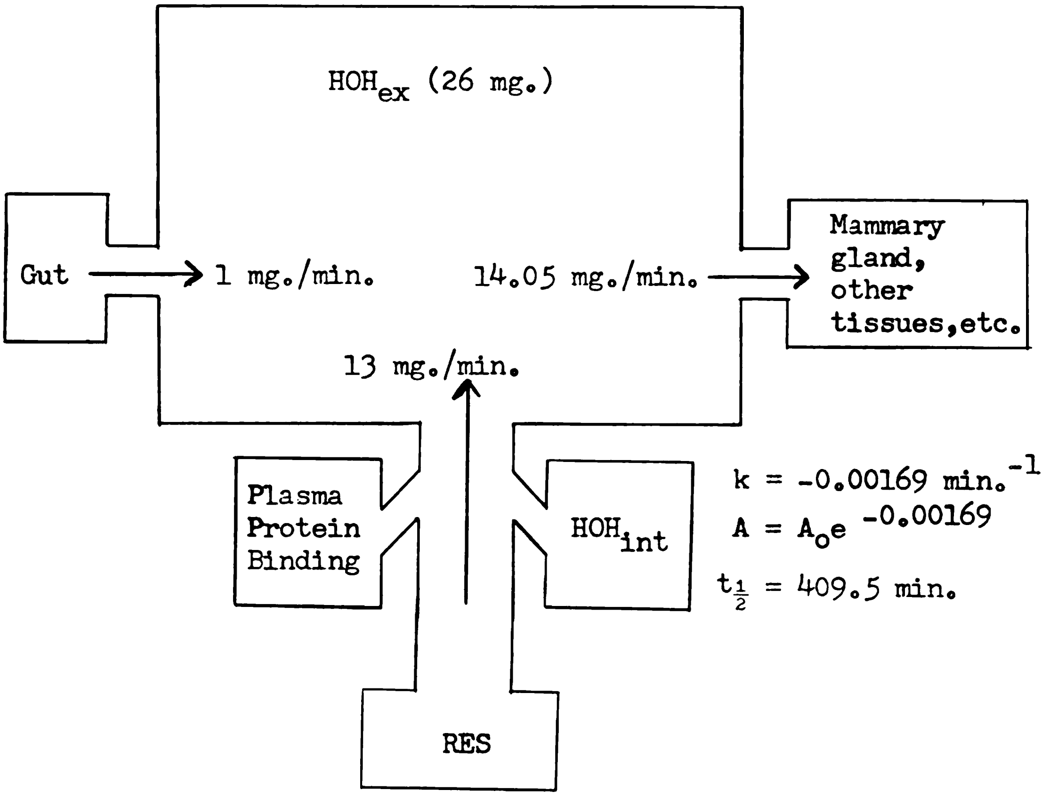
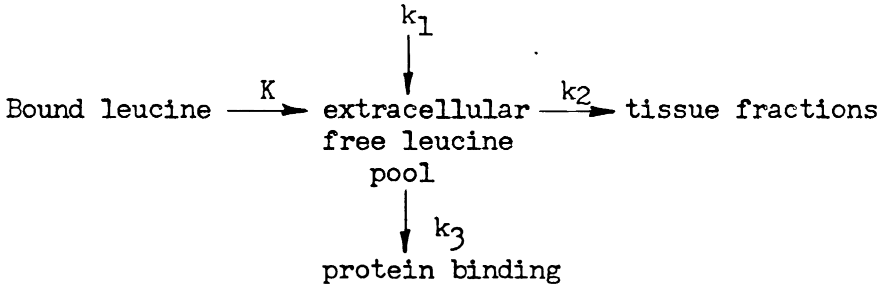


FIGURE 3. MODEL FOR THE TURNOVER OF THE EXTRACELLULAR POOL OF LEUCINE TWO HOURS POST INJECTION.

quantity available as a direct precursor of milk protein. The curves after 2 hours appear more like the curves for labeled amino acids administered orally or the curve expected when a colloidal suspension is injected and picked up by the reticulo-endothelial system. If this idea is correct, then a leucine depot must be postulated to account for the data obtained. It appears that a large percentage of this injected leucine was sequestered somewhere in the rabbit's body and was not available for milk protein synthesis. It was then turned over at a rate slightly less than the turnover rate of the extracellular leucine pool. It is postulated that likely sites for this leucine depot might be the reticulo-endothelial system, the intracellular leucine pool, and plasma protein binding. It is reasonable to expect that all of these possibilities might be involved. Such a system as this might be expressed in the following way:



The rate of tissue fraction labeling or turnover might simply reflect the constant (k), plus the biological steady state represented by $k_1 = k_2 + k_3 + k_n$. These rates would be referred to the rate of free leucine in the blood.

This discussion has been an attempt to explain and understand the kinetics of the data presented in Table 1 for the specific activities of free blood leucine in the rabbit. It was felt that this was necessary and that at least an attempt should be made to determine the parameters

for the forthcoming discussion on the precursors of milk proteins. Unfortunately, this discussion has been seriously hampered by the lack of data on the various pool sizes which make it impossible to completely analyze the curve and make some estimate of the complex exponential equations required to fit the curve presented for free blood leucine in Figure 1.

2. Decrease in the Specific Activity of Free Blood Leucine. Comparison of Results with Other Work

There are many reports in the literature dealing with the administration of labeled amino acids to animals. Many of these reports describe an extremely rapid uptake of the free amino acid from the blood. Unfortunately, none of these reports have provided a completely adequate answer to this problem.

Henriques *et al.* (1955) have reported similar results with glycine in rabbits. They have demonstrated a very high uptake of labeled glycine by the liver. They have subjected their curves to mathematical analysis and postulate at least a three-compartment curve for the early part of their curve as described by Solomon (1949). The organism does not distinguish between an isotopically labeled metabolite and the natural form of the compound. Since the data in Table 1 are presented as specific activity, a system or systems for the rapid uptake and replacement of free blood leucine must be postulated for the rapid decrease in specific activity of the free blood leucine. This has been attempted in the above discussion. There are a number of possibilities to explain this rapid change of the specific activity in free blood leucine. These are: very rapid uptake of leucine by the reticulo-endothelial system, mammary tissue, liver, spleen, kidney, lung, intestinal mucosa, interstitial

fluid, lymph, plasma proteins, and renal excretion. Friedberg <u>et</u> <u>al</u>. (1948) have reported that 15 minutes after the intravenous injection of S^{35} labeled methionine into dogs and rats, the activity of the free amino acid in kidney and spleen was higher than the activity of the free amino acid in the plasma. They also reported that the activities in liver and lung were about the same as the activity in plasma and that in 30 minutes the activity of methionine in the proteins of the intestinal mucosa was higher than the activity of free methionine in the plasma.

Niklas and Maurer (1952) administered S^{35} labeled methionine orally to rats and demonstrated a very rapid incorporation into the plasma proteins with a corresponding rapid decrease in the specific activity of the free methionine in blood plasma. Tarver and Schmidt (1942) have also shown a rapid decrease in the specific activity of free plasma S^{35} methionine when it was fed to dogs. Abdon and Tarver (1951a, 1951b) injected 10 mg. serine-β-C^{14} into Long-Evans strain male rats and found a very rapid decrease in the specific activity of free blood serine similar to the results in Table 1. Hughes (1954) has reported that there is a considerable loss of proteins from the blood due to leakage through the capillaries into the interstitial fluids. This interstitial fluid represents about 20% of the total volume of the mammalian organism or 3 times the volume of blood and thus represents a sizable pool for amino acids. In addition, Hughes states that studies with labeled plasma proteins have shown that these proteins are found in the interstitial fluids and lymph in a very short time and reach equilibrium within a few hours. He further states that the capillary permeability for the free amino acids is 100 times that of the blood proteins. This would bring the amino acids into equilibrium within minutes after the injection of an amino acid directly into the circulatory system. Some of the free amino acid would be returned to the general systemic circulation via the lymphatic system, which may, in part, explain why the rate decrease in specific activity decreases after a few hours.

The mammary gland must represent a sizable pool for free amino acids since the specific activity of leucine within caseins, β-lactoglobulin and α-lactalbumin is quite high. These values at 2 hours post injection are reported in Table 1 as 1.48, 2.50 and 1.20 μc./mm. of leucine, respectively. This indicates that there must be a considerable uptake of amino acids by the mammary gland in the rabbit. Hammond and Marshall (1925) reported that the average weight of rabbit mammary glands dehydrated with 95% ethyl alcohol from 40 lactating animals during the first 20 days postpartum was 105 gm. Assuming that the dehydration was 50%, then the fresh weight would be about 200 gm. If 20% of this weight is interstitial fluid, then 40 ml. of interstitial fluid would be present. Graham (1937), Shaw and Petersen (1938), Reineke _et al_. (1939) and others have shown that there is an arterio-venous difference of amino acids across the mammary gland. These workers have shown that there is an active accumulation of free blood amino acids by the lactating mammary gland. Therefore, the concentration of amino acids in the interstitial fluid of the mammary gland should be equal to or greater than the concentration of the corresponding amino acids in the blood. This would represent at least 1 mg. of free leucine.

Renal excretion of free leucine could play an important role in the reduction of the specific activity of free blood leucine, since the level of leucine in the blood at zero time is about 3 times the average value reported in Table 3 in the appendix. The activity of the urine counted at 2 hours post injection is not excessively high, as shown by

Table 5 in the appendix. However, it is possible that a considerable amount of activity must have been excreted via the urine during the 2-hour period prior to the counting of the first urine sample. This might account for a major portion of the decrease in activity during the first few minutes post injection. It is possible that the concentration of leucine in the glomerular filtrate exceeded the renal threshold for leucine by the rabbit kidney. Also, there is the possibility that the renal threshold for D-leucine is very low. If this is true, the D form would be selectively excreted and could account for a considerable decrease in the specific activity of free blood leucine. It is also possible that the threshold for L-leucine was not reached, since Beyer <u>et al</u>. (1946) have reported that the maximal rate of reabsorption in the kidney of the dog was not reached with loads as high as 26.5 mg./100 ml. of filtrate. Also, they reported that less than 2% of the filtered amino acid was excreted.

If it is assumed that the data for free blood leucine levels presented in Table 3 of the appendix represent an average free blood leucine level, the level of free blood leucine in the blood for the first few minutes post injection is 3 times the normal level. If this is so, the possibility for luxury consumption of leucine into the various pools mentioned previously exists. This possibility might also help explain the very rapid disappearance of the labeled leucine from the plasma. Judging from the specific activities of leucine incorporated into the milk proteins, caseins, β-lactoglobulin and α-lactoglobulin reported in Table 1, luxury consumption could not have played too great a role in the mammary leucine pool. That luxury consumption was not too important appears to be true, since the specific activities of the

leucine of these proteins decreases at a relatively constant rate, as shown by Figures 1, 4 and 5. If there was an excess of leucine "stored" in some form in the mammary gland available for future synthesis of these proteins, then it would be expected that the specific activity of leucine incorporated into caseins, β-lactoglobulin and α-lactalbumin would not decrease as rapidly as it does. However, there is some slight suggestion of luxury consumption, since the specific activity of leucine in these proteins does not decrease as fast as the specific activity of free blood leucine. This is especially evident in the case of α-lactalbumin (Figure 5) during the first 12 hours post injection. Table 1 presents these specific activities of leucine from α-lactalbumin as 1.20, 1.20 and 0.92 µc./mm. of leucine at 2, 6 and 12 hours post injection, respectively. The above discussion is based on the assumption that the free blood leucine is the direct precursor of the leucine in casein, β-lactoglobulin and α-lactalbumin. It appears that luxury consumption is not a very important factor as far as the mammary gland is concerned. However, this does not exclude the possibility that luxury consumption of the injected leucine could be a very important factor in other organs which have been shown to take up large amounts of free blood amino acids, such as the liver, kidney, spleen, lungs, and intestine.

Since the decrease in the specific activity of free blood leucine is so great during the first few minutes, it seems reasonable to feel that all of the mechanisms discussed above play an important role in the removal of free leucine from the blood. It is felt that the role of the mammary tissue, reticulo-endothelial system, plasma protein binding, kidney, spleen and liver are of special importance.

There are only two possible ways that the specific activity of free blood leucine can be reduced. They are selective removal of the

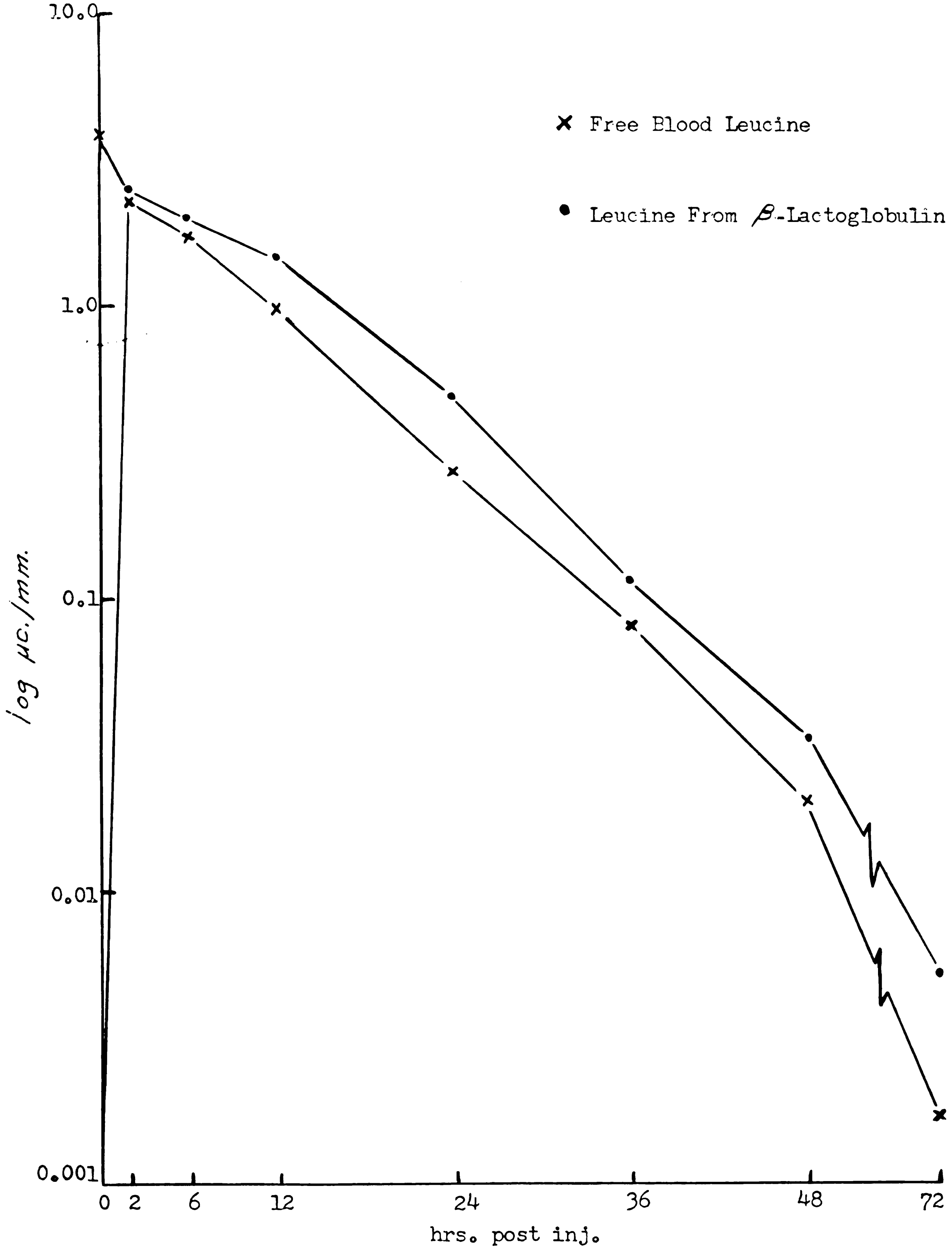


FIGURE 4. THE LOG OF THE SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM β-LACTOGLOBULIN ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

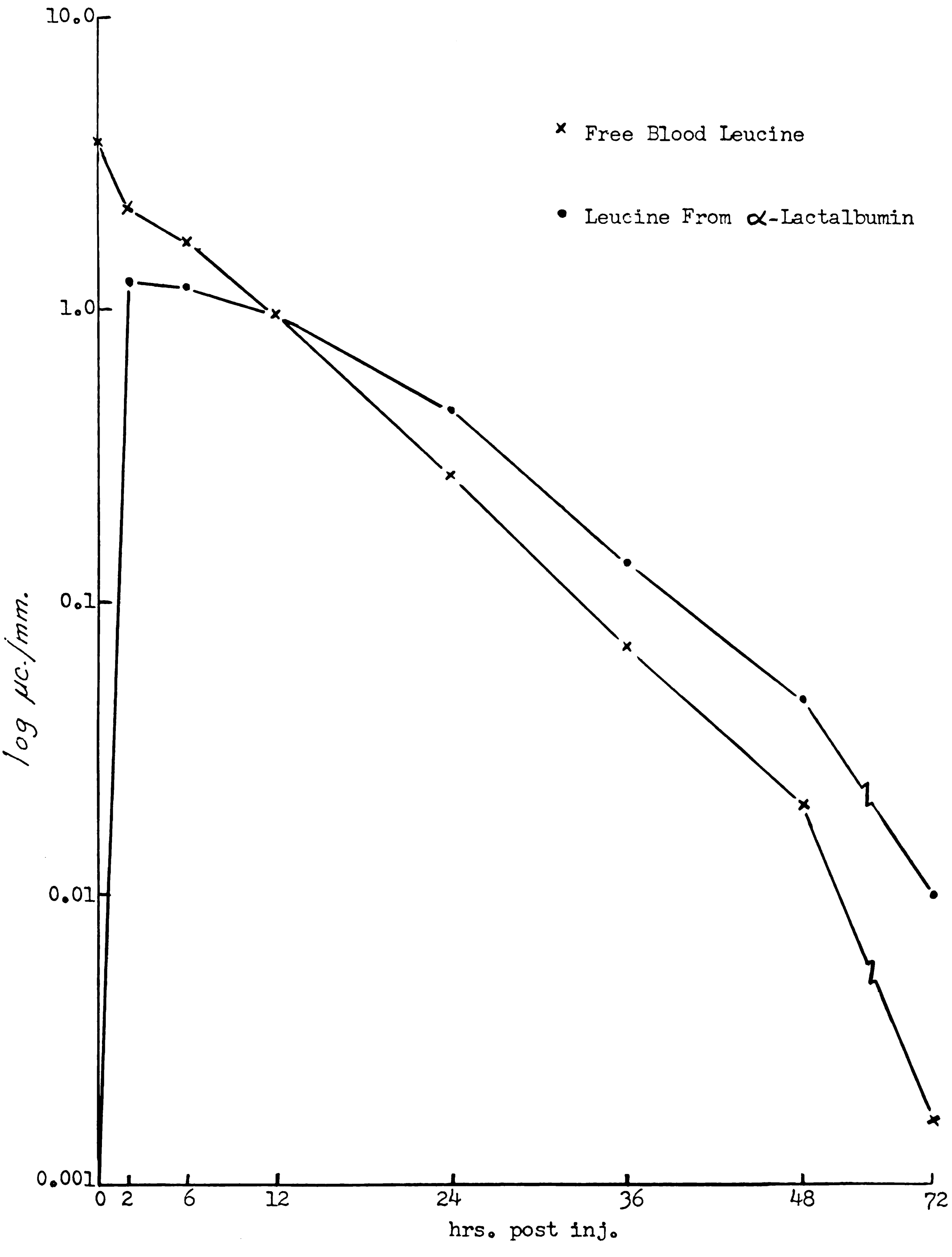


FIGURE 5. THE LOG OF THE SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM α-LACTALBUMIN ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

radioactive leucine and no removal of the non-labeled leucine or dilution of the labeled leucine of the blood with non-labeled leucine. The only possibility for the first pathway is selective excretion of D-leucine by the kidney. At best, this could only account for a 50% decrease in the specific activity if all of the D form remained in the blood and was 100% excreted by the kidney. This ideal situation is not possible in a biological system such as this. The other possibility is absorption of free blood leucine into tissues and organs and absorption of dietary leucine from the gut at an extremely fast rate as discussed previously. This is based on the assumption that the level of free blood leucine remains relatively constant. Also, the return of non-labeled leucine to the general systemic circulation from the lymph during the first minutes would have a dilution effect and should be taken into consideration. Humphrey and Sulitzeanu (1958) have shown that the rate of exchange of amino acids between cells and tissue fluids is of the order of 100 mg./min. and that the size of the intracellular amino acid pool in which this rapid exchange occurs is of the order of 100 times the plasma free amino acid pool. This could most certainly account for a very sizable and rapid dilution of the injected DL-leucine-2-C^{14}. Considering all of the above discussion, it is possible to account for this extremely rapid decrease in the specific activity of free blood leucine. However, experiments should be designed to determine what is responsible for this initial rapid decrease in the specific activity of an injected amino acid. The kinetics of this phenomena are not clearly understood at this time.

3. Free Blood Leucine as a Direct Precursor of Caseins, β-Lactoglobulin and α-Lactalbumin of Milk

Examination of the curves presented in Figures 1, 4 and 5 for the specific activities of leucine from caseins, β-lactoglobulin and α-lactalbumin versus time, respectively, show a marked similarity and follow the specific activity of free blood leucine quite well. This is especially true in the case of β-lactoglobulin. Zilversmit *et al.* (1943) have developed the general precursor, product relationships. The theoretical curves developed by these workers provide the criteria for decision as to whether the precursor measured is the direct precursor of the product. These criteria are: The specific activity of the precursor must exceed the specific activity of the product until the specific activity of the product reaches its maximum, at which time the two specific activities become equal and thereafter the specific activity of the product is greater than the specific activity of the precursor. The theoretical curves for precursor and product as outlined by Zilversmit *et al.* (1943) are different from the curves presented in Figures 1, 4 and 5. This is to be expected because the product is constantly being removed from the system and there is no turnover rate for these products. These three curves fit the criteria for precursor and product quite well. The curve for β-lactoglobulin matches the criteria almost perfectly. When the area under the blood curve is compared with the area under the milk protein curve, it becomes evident that the areas are quite similar. If only one component in the blood is significantly labeled and it is acting as a direct precursor of the milk protein, then the area under the specific activity curve of free blood leucine should equal the area under the specific activity curve of leucine from the milk proteins. Dividing the area under the free leucine of blood curve into the area

under the milk curve should give the percent of leucine in the milk protein derived from the free leucine of blood plasma. In these 3 cases, it is evident that this would give a value greater than 100%. This is attributed to a time lag. From inspection of these curves it becomes evident that the specific activity of leucine in the milk protein is equal to the specific activity of the free blood leucine 2 to 6 hours earlier. An example may help to clarify this theory. The specific activity of the leucine of β-lactoglobulin (Figure 4) at 32 hours post injection is 0.2 µc./mm.; at 28 hours post injection, the specific activity of free blood leucine is also 0.2 µc./mm. This can be interpreted to mean that it takes an average of 4 hours for leucine to move from the plasma pool to the free leucine pool in the mammary gland and then synthesized into one of these milk proteins and then into the lumen or duct, ready to be excreted as a milk constituent. Also, the high values could be due, in part, to residual milk left in the lumen of the alveoli after milking, which would tend to cause higher than actual values at the next milking. This might also explain the irregularities observed in some of the curves presented.

Barry (1952) has carried out similar experiments on lactating goats. He has injected goats with (P^{32}) Na_3PO_4 and determined the specific activity in blood and casein at various time intervals. The work of Aten and Hevesy (1938) and Colas et al. (1950) showed conclusively that all of the phosphorus in casein is derived from free phosphate of the blood. Barry reasoned that the areas under these two curves for blood phosphorus and casein bound phosphorus should be equal. When he divided the areas, he found that the experimental value was 70% instead of the theoretical 100%. Therefore, he has reported that this treatment of the data will give a value within ±30% of the true value. If Barry's work is correct, then it can be concluded that most of the leucine in caseins, β-lactoglobulin and α-lactalbumin synthesized by this rabbit was derived directly from the free leucine in the blood; also, that the leucine in tha mammary pool is derived from the leucine in the blood and is not contributed to by other sources to any major extent.

Barry (1952, 1956), Sheldon-Peters and Barry (1956), Sansom and Barry (1958), and Barry (1958) have obtained evidence by similar experiments on goats that lysine, tyrosine, glutamine, glutamic acid, asparagine and proline of goats' casein is derived to a large extent from the corresponding free amino acid or amide of the blood. They have also shown that at least 70% of the essential amino acids and at least 50% of the non-essential amino acids are derived from the corresponding free amino acids of the blood. They have further shown that a very small proportion of the non essential amino acids are derived from glucose within the mammary tissue. Campbell and Work (1952), Askonas _et al._ (1954), and Askonas _et al._ (1955) have done similar experiments on the rabbit and goat. These workers have shown that the valine, lysine, glycine, and methionine of casein and β-lactoglobulin are derived directly from the blood. They have also shown that the plasma protein activity is approximately 10% of the activity present in casein and β-lactoglobulin at 6 hours post injection in the rabbit and goat. They have also been able to demonstrate that the labeling in these proteins was uniform by partial hydrolysis, isolation of the various peptides and counting these peptides.

Larson and Gillespie (1957) have injected (C^{14}) sodium carbonate into a cow and measured specific activities in the milk proteins. They found high specific activities in α- and β-casein, β-lactoglobulin and α-lactalbumin which were about equal. This suggests, also, that these proteins were formed from free amino acids in the blood. Kleiber _et al._

(1952) have also reported similar results. It has been shown by this work that the leucine incorporated into rabbit milk caseins, β-lactoglobulin and α-lactalbumin was derived from leucine that existed some hours earlier as free blood leucine. These results are in very good agreement with the results of others on other amino acids and other lactating mammals.

4. The Relationship Between γ-Globulins of the Blood and the Immune Globulins of Milk

Inspection of the curves for the specific activity of leucine from γ-globulins of the blood and immune globulins of the milk presented in Figures 6, 7 and 8 indicate a remarkable similarity between them. They are almost identical and there is no significant difference between them. It is quite obvious that the curve for immune globulins from milk differs to quite an extent from the curves presented for caseins, α-lactalbumin and β-lactoglobulin. This indicates that free blood leucine could not have been a direct precursor of leucine in immune globulins from milk since it has been pointed out in the previous section that leucine of caseins, α-lactalbumin and β-lactoglobulin are derived from this source. These data on blood γ-globulins and immune globulins indicate that the relationship between them is not that of a precursor and product since they do not meet the criteria of Zilversmit _et al._ (1943). It appears that these two protein fractions are extremely similar when judged on the basis of the specific activity of leucine incorporated into them by the lactating rabbit.

Table 2 and Figure 9 present the ratios of the specific activities of leucine from milk immune globulins to the specific activities of blood γ-globulins from samples taken from the animal at the indicated times

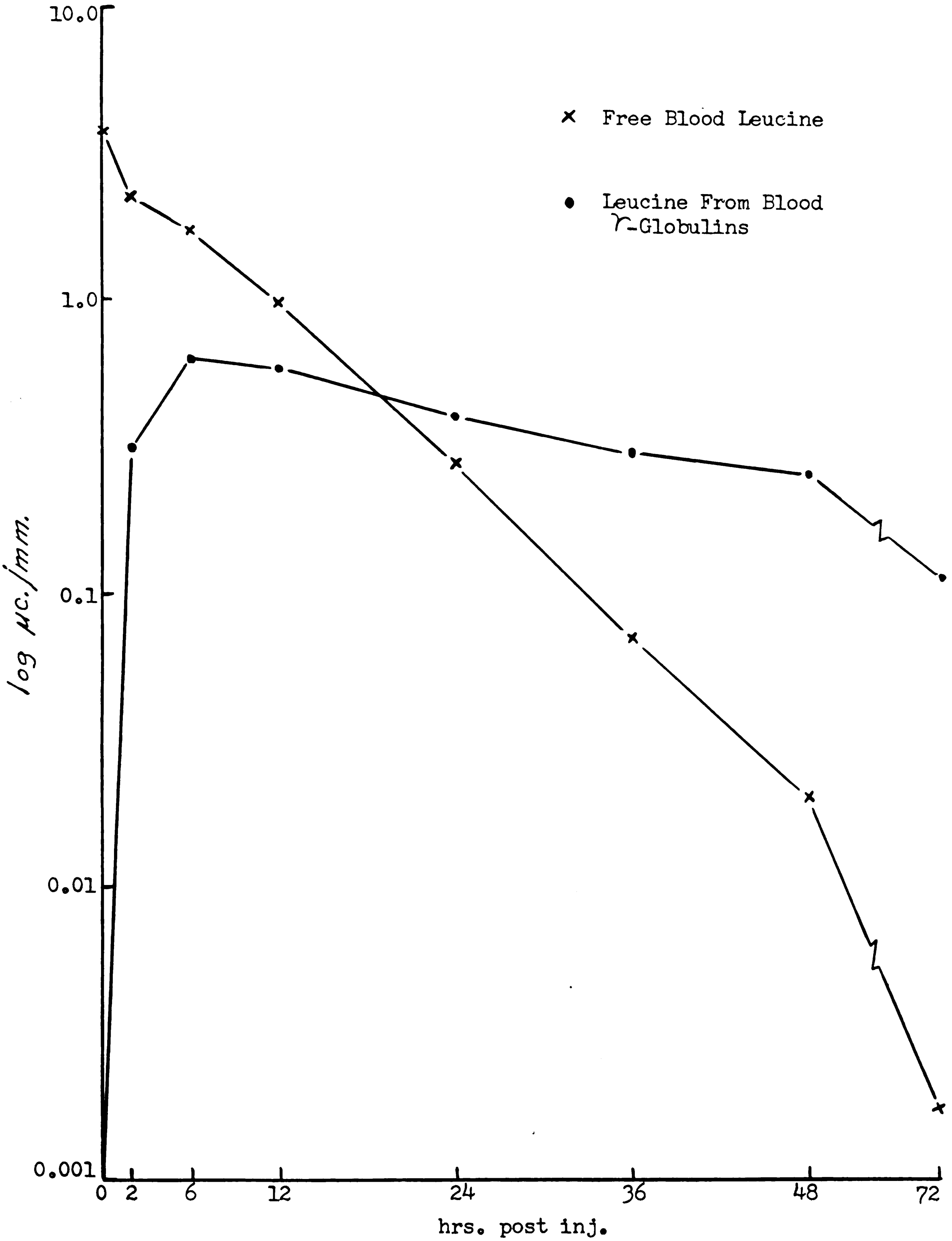


FIGURE 6. THE LOG OF THE SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM BLOOD γ-GLOBULINS ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

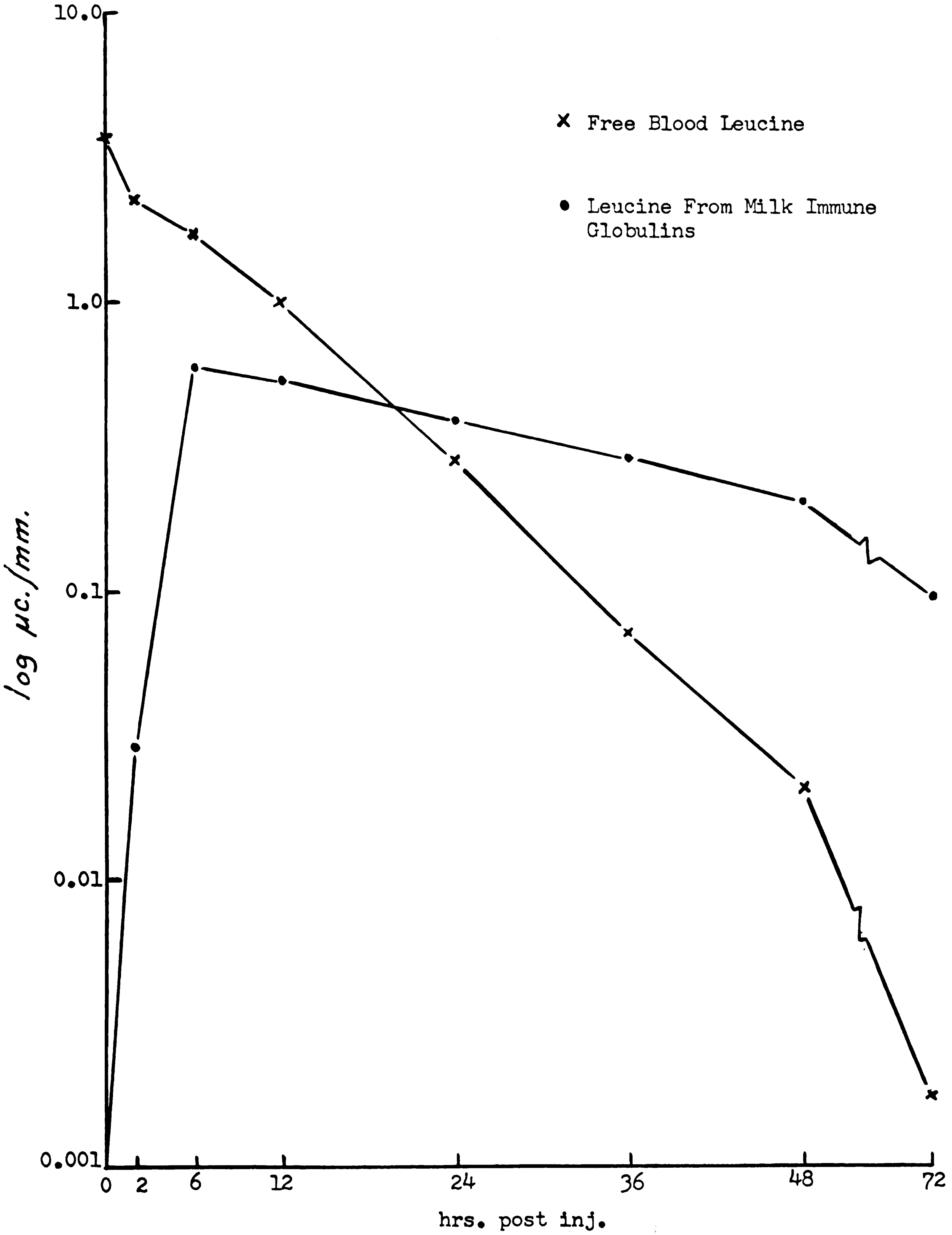


FIGURE 7. THE LOG OF THE SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM MILK IMMUNE GLOBULINS ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

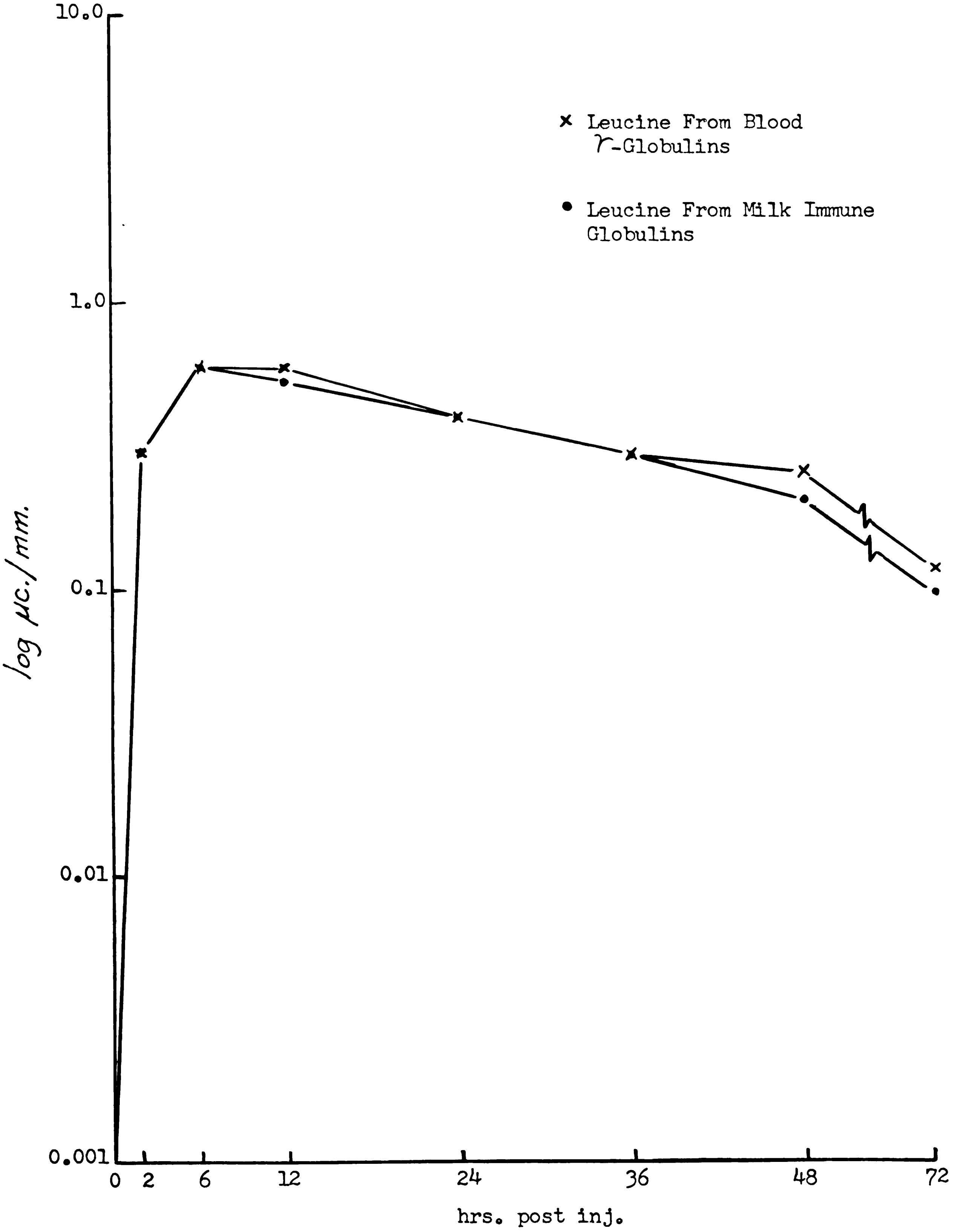


FIGURE 8. THE LOG OF THE SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR LEUCINE FROM BLOOD γ-GLOBULINS AND LEUCINE FROM MILK IMMUNE GLOBULINS ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

TABLE 2. RATIOS OF THE (a) SPECIFIC ACTIVITIES OF LEUCINE FROM MILK IMMUNE GLOBULINS TO THE (b) SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD γ-GLOBULINS FROM LACTATING RABBIT (NO. X-35, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 μC. OF LEUCINE-2-C^{14}.

hrs. post inj.	a/b	% of milk immune globulins derived from blood γ-globulins
2	0.916	91.6
6	0.959	95.9
12	0.906	90.6
24	0.966	96.6
36	0.977	97.7
48	0.791	79.1
72	0.837	83.7
	0.909 ± 0.174[1]	

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[1] The mean of the ratios is presented with standard error.

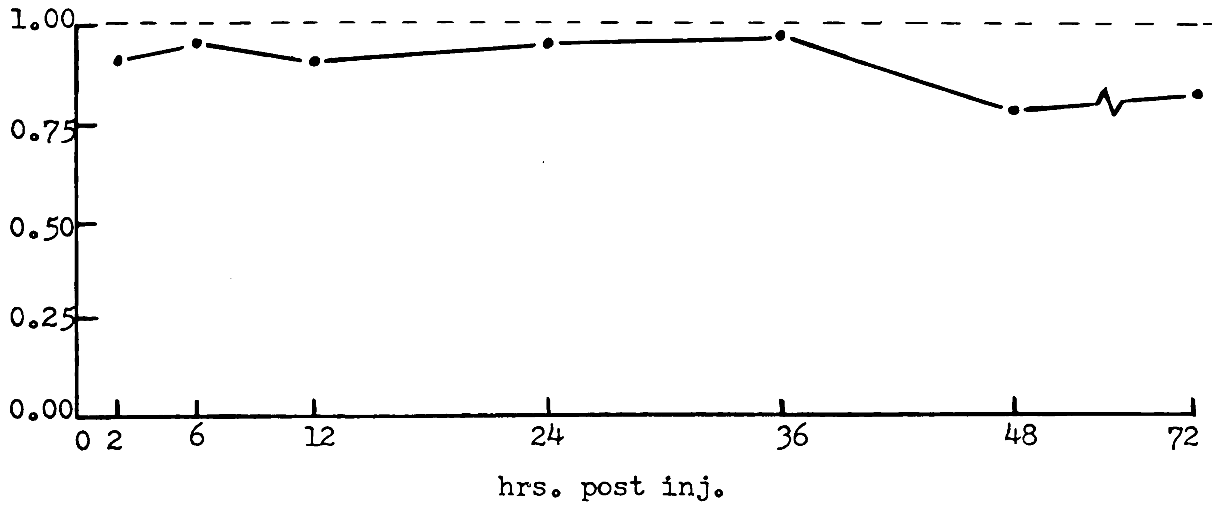

FIGURE 9. RATIOS OF THE SPECIFIC ACTIVITIES OF LEUCINE FROM MILK IMMUNE GLOBULINS TO THE SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD γ-GLOBULINS ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

post injection of leucine-2-C^{14}. These data indicate that the system was probably in equilibrium at 2 hours post injection, the time when the first milk sample was taken. The average of these ratios is 0.909 ± 0.174. This indicates that during the period of this experiment, at least 73.5% of the immune globulins secreted into the milk by this animal came directly from blood γ-globulin. The data presented in Table 2 indicate an efficient mechanism for the transport of this protein fraction from the vascular system to the interstitial fluid and then into the epithelial cells of the mammary gland and thus into the milk. This is indicated because the system is already in equilibrium sometime prior to 2 hours post injection. An efficient mechanism for transport of γ-globulin is also indicated by the relatively high molecular weight of rabbit blood γ-globulin of 160,000, which has been determined by Nichol and Deutsch (1948). The data indicate very strongly that the immune globulins were not synthesized within the mammary gland. This is supported by the findings of Humphrey and Sulitzeanu (1958), who injected (C^{14}) amino acids into rabbits to locate the sites of synthesis of antibodies. They found labeled antibodies in lung, bone marrow, spleen, and lymph nodes. They did not find labeled antibodies in liver, kidney, or mammary tissue. Askonas and Humphrey (1958) have shown that cells from various tissues are capable of synthesizing antibodies and other γ-globulins *in vitro*. The spleen, lung, and bone marrow were shown to be the most important sites of synthesis.

There is voluminous literature on the immune globulins of bovine milk. Ehrlich (1892) was the first to detect antibodies in colostrum. Smith (1946a, 1946b, 1946c, 1946d, 1947) has done a tremendous amount of work on the physical characteristics and chemical composition of milk immune globulins and blood γ-globulins. He has been able to demonstrate quite a close relationship between the two protein fractions but has shown some differences in amino acid composition. This is particularly true in the case of leucine. Smith (1948) reported in his review on immune globulins in cow's milk that his average values for the percent of leucine in euglobulins, pseudoglobulins and γ-globulins were 10.4, 9.1 and 7.4, respectively. This is approximately a 20% difference between immune globulins of cow's milk and γ-globulins. This would mean that a considerable amount of leucine is incorporated into the γ-globulins of blood by the mammary gland prior to their secretion in the milk. The data in Table 1 do not substantiate this process in the case of the lactating rabbit. However, it is probable that the specific activity of free leucine in the mammary gland and the specific activity of free leucine being utilized for synthesis of γ-globulins at other sites in the body are very similar. If this is true, then determinations of specific activity of leucine will not provide information on the incorporation of additional leucine into γ-globulins by the mammary gland. There are inherent difficulties in amino acid determinations on protein hydrolysates which make it difficult to state positively that these proteins were identical. Hansen et al. (1947) have also shown a close similarity between the amino acid composition of a purified pseudoglobulin and human γ-globulin.

Studies by Jameson et al. (1942), San Clemente and Huddleson (1943) and by Hansen and Phillips (1947) have shown conclusively by electrophoresis that the blood of the newborn calf is deficient in the γ-globulin fraction. Hansen and Phillips (1947) and Hansen et al. (1947) have shown that this γ-globulin appears very rapidly in the blood of the newborn calf if colostrum is fed during the first 24 hours postpartum. These electrophoretic studies demonstrated very similar mobilities for the blood γ-globulins and the immune globulins of milk. More recent studies by Larson and Kendall (1957) by quantitative electrophoresis have shown that the β_2- and γ_1-globulins build up in the blood stream several weeks prior to parturition and then drop significantly as the colostrum is being formed. Larson (1958) has shown by quantitative electrophoresis that the immune globulins found in the mammary secretions can account for 85 to 107% of the β_2- and γ_1-globulins lost from the blood.

Studies with isotopes by Campbell and Work (1952), Kleiber _et al._ (1952), Black and Kleiber (1954), Askonas _et al._ (1954), and Larson and Gillespie (1957) have indicated that there are two types of labeling which occur in milk proteins in the lactating bovine, goat and rabbit; high specific activities which indicate that these proteins are derived from free amino acids of the mammary pool which is in equilibrium with the blood free amino acid pool, and the other type of labeling is a low specific activity in the order of magnitude of one-tenth that of the high activity. The data of these workers suggested that the low activity fraction consists primarily of immune globulins and that it comes directly from the blood without alteration in the mammary gland. Askonas _et al._ (1954) immunized a rabbit with formalin-treated pneumococcus type III. The animal was then injected with 25 µc. of S^{35} labeled DL-methionine. He isolated the antibodies from milk and blood samples by precipitation with the antigen. He found a very close correlation between the activities in the antibodies from milk and the antibodies from blood. However, the activities of their samples were very low. They were from 7 to 17 counts/min./0.3 sq. cm. of infinite thickness. These activities are so low that it is difficult to evaluate the significance of these data and to state positively that they were derived from the same source. The data in Table 1 show that the counts of the milk immune globulins and blood γ-globulins are in the order of thousands of counts per minute and are therefore much more significant.

Many workers have demonstrated the striking similarity between blood γ-globulins and milk immune globulins in the bovine. Larson (1948) in particular has demonstrated the quantitative relationship between the two in the bovine. The data presented in Table 1 and Figures 6, 7, 8 and 9 show quantitatively that at least 73.5% of the immune globulins of rabbit milk are derived directly from the blood γ-globulins. These data further show that the milk immune globulins were not synthesized within the mammary cells because of the dissimilarity between the specific activities of leucine from milk immune globulins and the specific activities of leucine from casein, β-lactoglobulin and α-lactalbumin which have been shown to come directly from the free amino acid pool of the mammary gland which is in equilibrium with the free amino acid pool of the plasma. Furthermore, the results on the rabbits are in agreement with the results obtained on the bovine. They also substantiate the work of Askonas _et al._ (1954) on the rabbit and are believed to be more reliable due to the much higher level of activity. Also, it has been possible to quantitate these data and they agree quite well with the results obtained by Larson (1958) on the cow.

5. The Origin of "Blood" Serum Albumin in Rabbit Milk

Table 1 presents the data on the specific activities of leucine from "blood" serum albumin from milk and blood albumin. Again, there is a very close resemblance between the labeling in these two proteins.

Figures 10, 11 and 12 demonstrate graphically the extremely close similarity between them. This case appears to be the same as the situation between blood γ-globulin and immune globulins from milk. On the basis of these data, it appears that the blood albumin and "blood" serum albumin of milk are identical since their activities are so similar. Table 3 and Figure 13 present the ratios of the specific activities of leucine from "blood" serum albumin from milk to the specific activities of leucine from blood albumin. They show that the ratios are quite close to one. The mean of the ratios indicates that at least 77.4% of the "blood" serum albumin in milk from this rabbit comes directly from the blood albumin during this 72 hour period. The equilibrium was reached very rapidly. From the data, it appears that it was reached in less than 2 hours. These results are in very close agreement with the results for immune globulins from milk and blood γ-globulin.

This "blood" albumin from rabbit milk has not been reported previously in the literature. These are the first data of either a qualitative or quantitative nature on "blood" albumin from rabbit milk. Larson and Gillespie (1957) have reported this protein in bovine milk to be the same as blood albumin. Earlier, Polis et al. (1950) reported a method for the separation of "blood" serum albumin from cow's milk and reported that its physical characteristics were the same as blood albumin. Also, Coulson and Stevens (1950) have shown that milk and blood albumin of the cow are serologically identical. These data on "blood" serum albumin from rabbit milk are in good agreement with the data on the cow.

6. The Specific Activity of Leucine Incorporated into the α- and β-Globulins of Blood

Table 1 and Figures 14 and 15 also present the specific activity in the α- and β-globulins of the blood. The activity is of the same

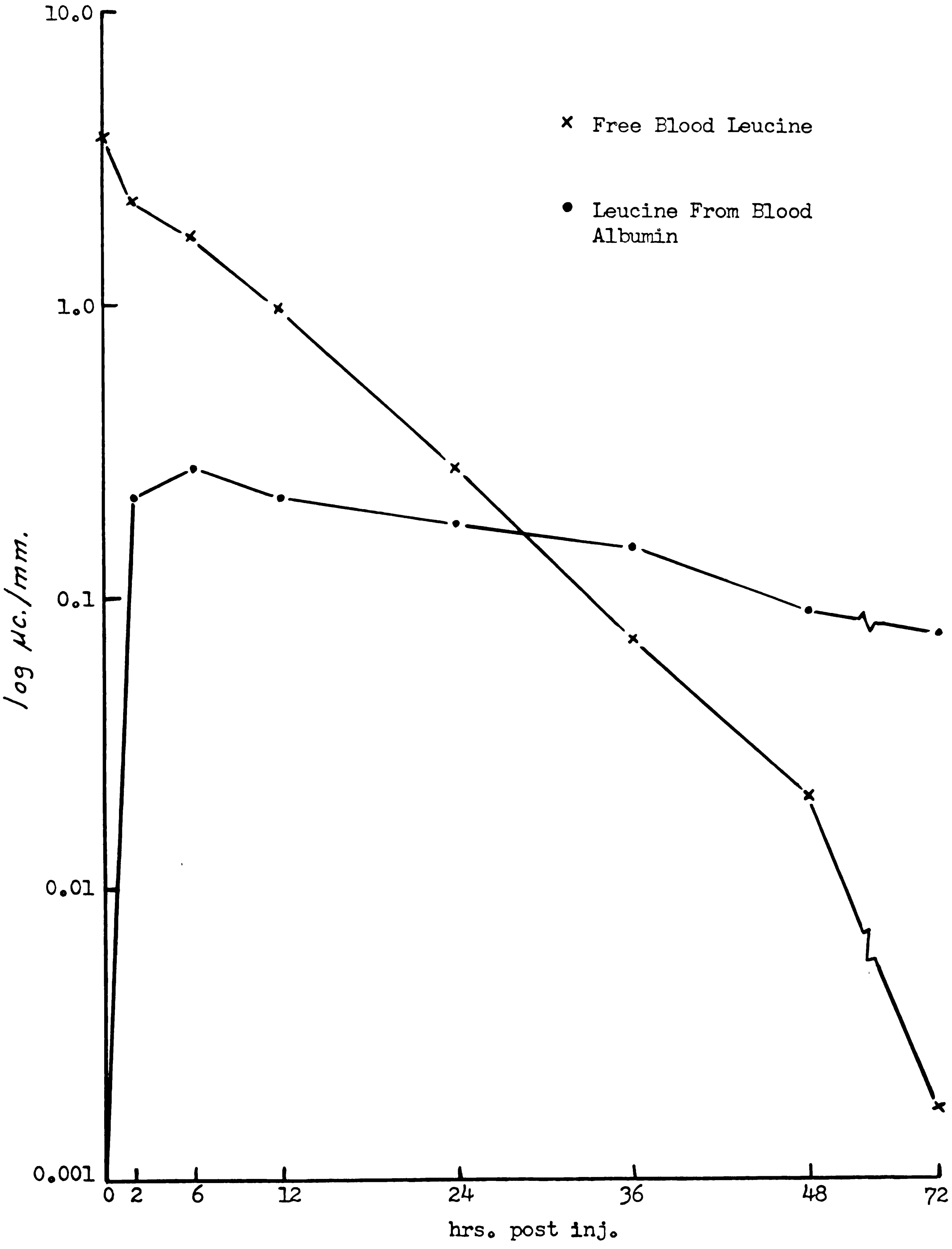

FIGURE 10. THE LOG OF THE SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) OF FREE BLOOD LEUCINE AND LEUCINE FROM BLOOD ALBUMIN ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

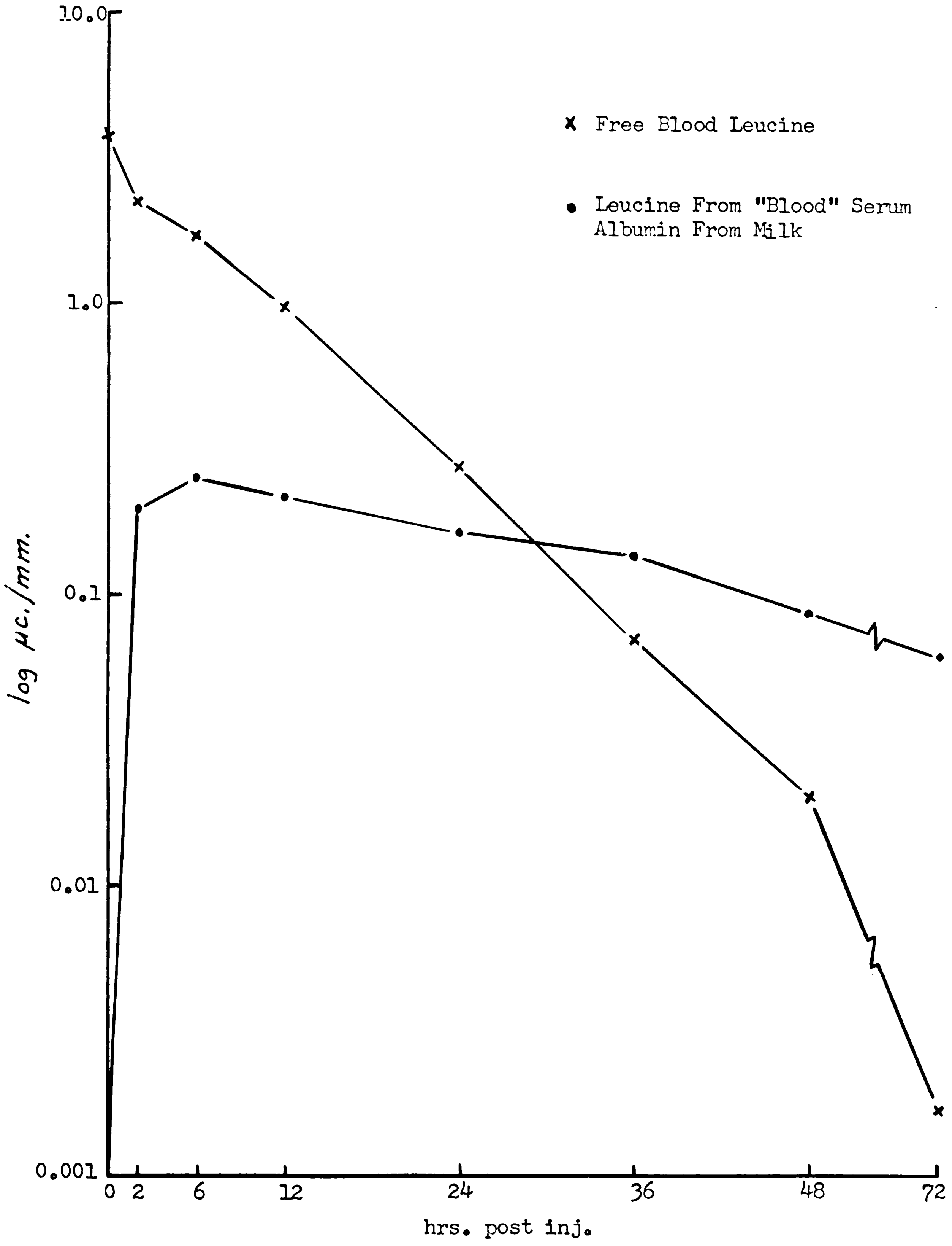

FIGURE 11. THE LOG OF SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) OF FREE BLOOD LEUCINE AND LEUCINE FROM "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

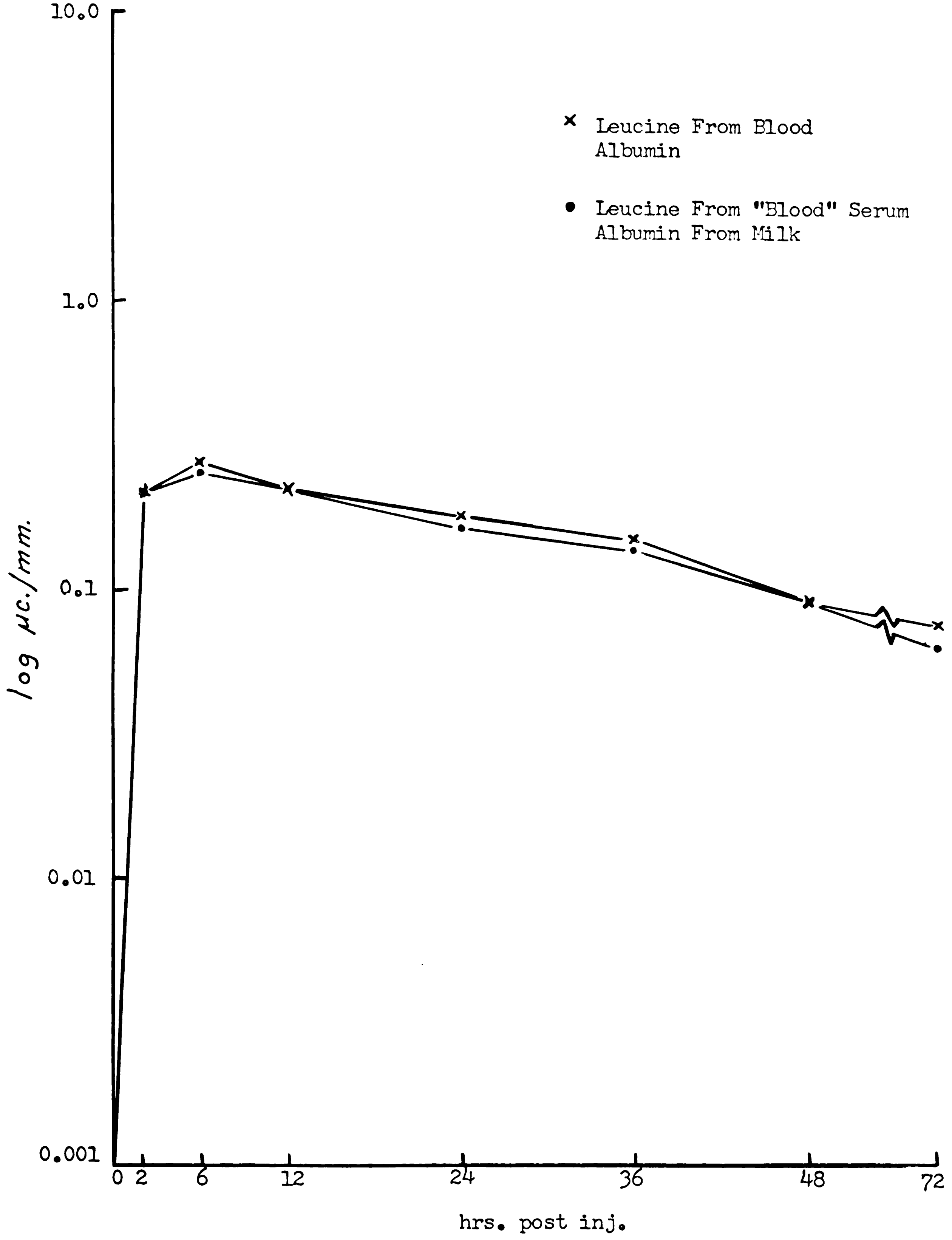

FIGURE 12. THE LOG OF THE SPECIFIC ACTIVITY (μc/mm.) VS. TIME (hrs. post inj.) OF LEUCINE FROM BLOOD ALBUMIN AND LEUCINE FROM "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

TABLE 3. RATIOS OF THE (a) SPECIFIC ACTIVITIES OF LEUCINE FROM "BLOOD" SERUM ALBUMIN FROM MILK TO THE (b) SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD ALBUMIN FROM LACTATING RABBIT (NO. X-35, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 µC. OF LEUCINE-2-C^{14}.

hrs. post inj.	a/b	% of "blood" serum albumin from milk derived from blood albumin
2	0.900	90.0
6	0.917	91.7
12	1.031	100.0
24	0.921	92.1
36	0.920	92.0
48	0.967	96.7
72	0.854	85.4
	0.916 ± 0.142[1]	

[1] The mean of the ratios is presented with standard error.

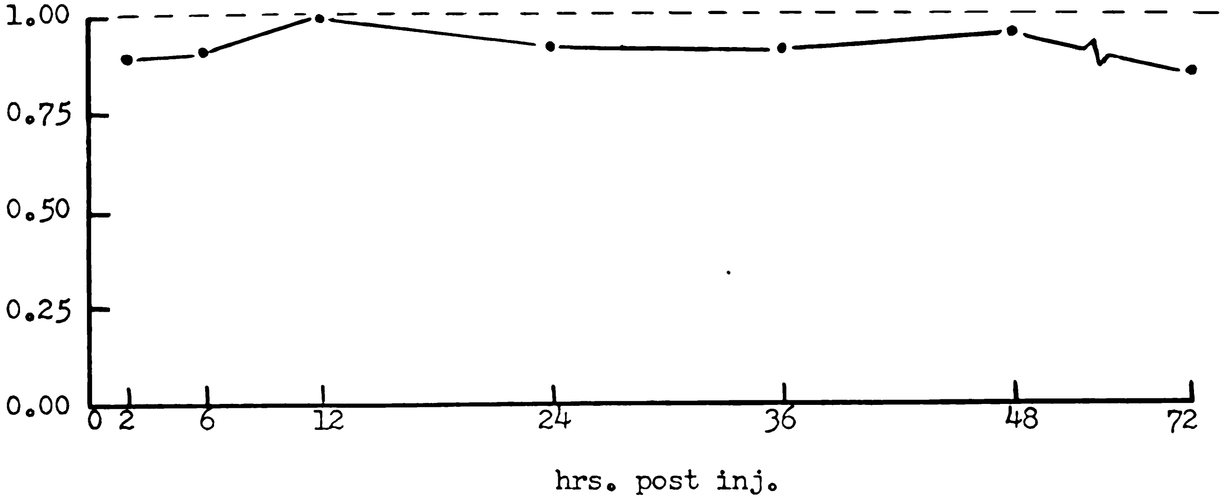

FIGURE 13. RATIOS OF THE SPECIFIC ACTIVITIES OF LEUCINE FROM "BLOOD" SERUM ALBUMIN FROM MILK TO THE LEUCINE FROM BLOOD ALBUMIN ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POST-PARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

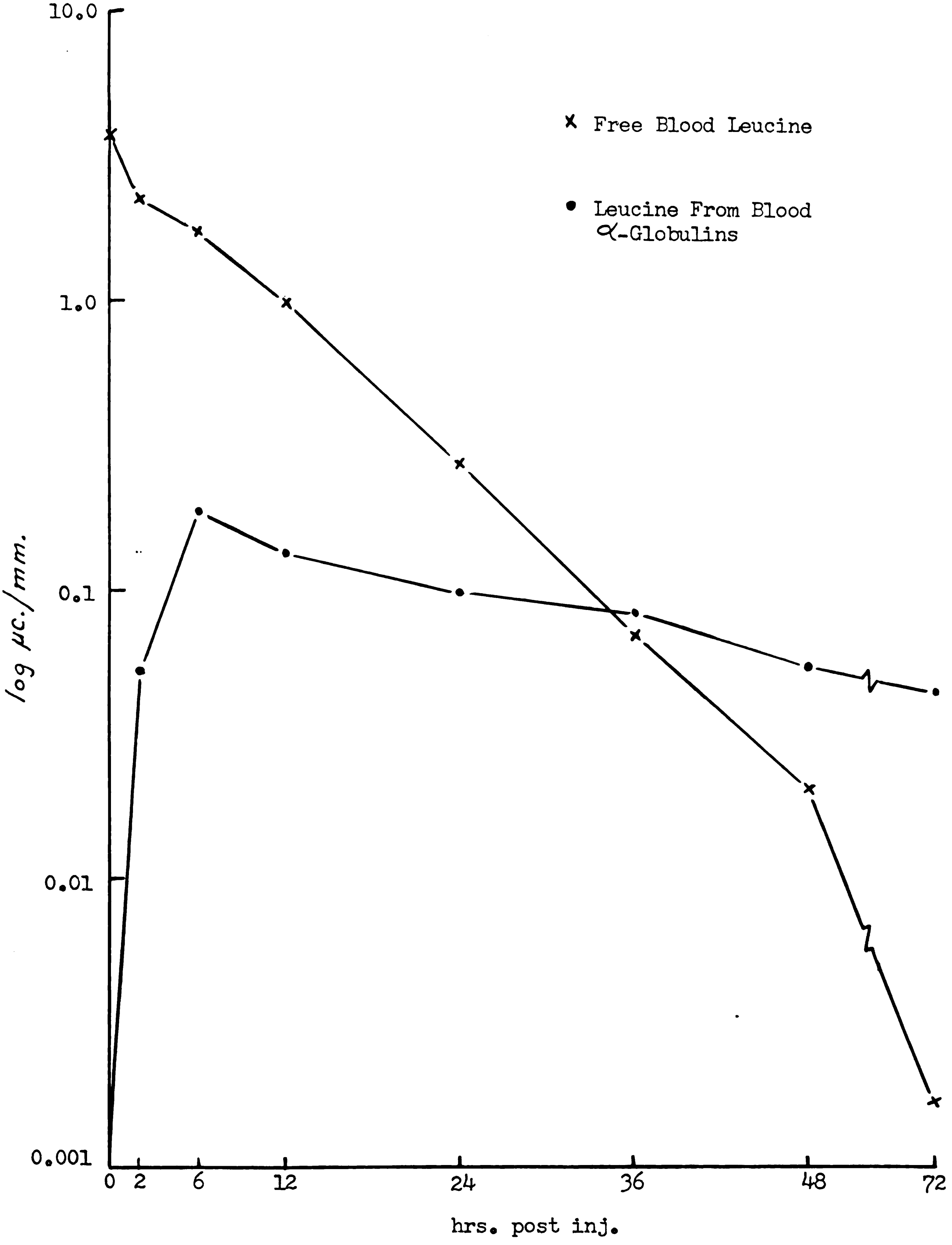

FIGURE 14. THE LOG OF SPECIFIC ACTIVITY (µc./mm.) VS. TIME (hrs. post inj.) OF FREE BLOOD LEUCINE AND LEUCINE FROM BLOOD α-GLOBULINS ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 µC. OF DL-LEUCINE-2-C^{14}).

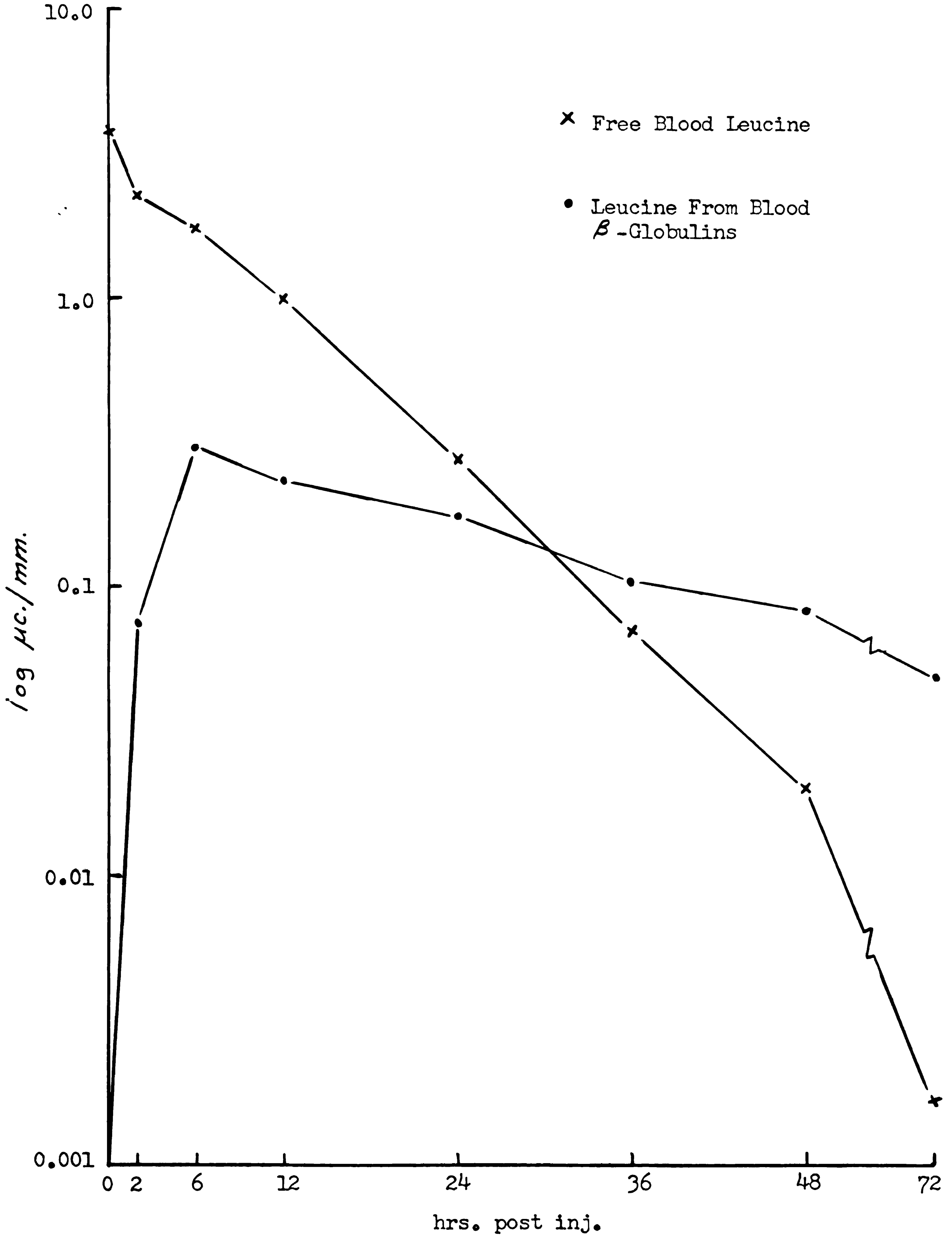

FIGURE 15. THE LOG OF SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) OF FREE BLOOD LEUCINE AND LEUCINE FROM BLOOD β-GLOBULINS ISOLATED FROM X-35 (LACTATING RABBIT 14TH-17TH DAY POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

order of magnitude as the other blood proteins discussed previously. Here again the activity reaches its maximum in about 6 hours after the injection of the DL-leucine-2-C^{14}. On the basis of the data presented in Table 1, it does not appear that these proteins of blood make a significant contribution to any of the rabbit milk proteins discussed above. This is true because the specific activity of leucine from these proteins is only a fraction of the specific activity of leucine from caseins, β-lactoglobulin and α-lactalbumin. Also, it is different from the specific activity of leucine from immune globulins of milk and "blood" serum albumin of milk.

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B. Experiments 2, 3 and 4. The Incorporation of Leucine-2-C^{14} and Glutamic Acid-2-C^{14} into Serum and Milk Proteins by Lactating Rabbits

Tables 4, 5 and 6, and Figures 16 through 28 present the data on a duplicate experiment with DL-leucine-2-C^{14} to substantiate the results reported in Tables 1, 2 and 3. It can be readily seen by a comparison of the tables and figures that the results are the same. The variations between the levels of activity between the two experiments may be ascribed to individual variations between the two animals. The discussion of and conclusions drawn from Tables 1, 2 and 3 are substantiated by the data of this duplicate experiment.

Tables 7 through 12 and Figures 29 through 54 present data on similar experiments when DL-glutamic acid-2-C^{14} was injected instead of DL-leucine-2-C^{14}. The same conclusions may be drawn from these data on the incorporation of glutamic acid into milk and blood proteins.

Leucine and glutamic acid were chosen for this work because they are both present in milk proteins in high concentration. Espe and Smith (1952) give values for casein and milk globulins as 9.3-10.5% and 18.7%, respectively, for leucine and 20.0-21.8% and 8.5%, respectively, for glutamic acid. Also, leucine is an essential amino acid and glutamic acid is a non-essential one. It is, to a certain extent, surprising that the quantitative results for these two amino acids agree so well as far as their incorporation into caseins, β-lactoglobulin and α-lactalbumin are concerned. The data on these three protein fractions indicate a higher level of incorporation than the data of Barry (1956) on the lactating goat.

TABLE 4. SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-32, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 μC. OF LEUCINE-2-C^{14}

hrs. post inj.	DPM	mg. leucine counted	μc./mm. x 10^2
		Free Blood Leucine	
0.16	11,790	0.20	350.00
2	4,655	0.15	186.00
6	1,200	0.15	47.00
12	480	0.15	19.00
24	210	0.20	6.20
36	70	0.20	2.00
48	21	0.20	0.62
72	5	0.20	0.15
		Leucine From Casein	
2	191,100	4.26[1]	265.00
6	117,450	4.41	157.00
12	11,700	4.05	17.00
24	4,860	4.42	6.49
36	1,820	4.00	2.69
48	819	3.90	1.34
72	242	4.31	0.33
		Leucine From Milk β-Lactoglobulin	
2	42,100	0.95	262.00
6	146,995	5.40	162.00
12	15,090	1.97	45.00
24	2,447	2.46	5.88
36	952	3.40	1.65
48	435	4.48	0.57
72	202	3.93	0.30
		Leucine From Milk α-Lactalbumin	
2	65,550	2.20	175.00
6	28,650	1.93	87.70
12	39,852	4.12	57.10
24	11,810	2.88	23.20
36	4,649	3.05	9.00
48	3,183	3.89	4.80
72	218	2.35	0.55

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[1] The count of this sample is not significantly different from background.

TABLE 4. (CONT.) SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-32, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 μc. OF LEUCINE-2-C^{14}

hrs. post inj.	DPM	mg. leucine counted	μc./mm. x 10^2
Leucine From Milk Immune Globulins			
2	8,064	2.63	18.10
6	23,589	2.87	48.40
12	8,762	1.34	38.50
24	12,685	2.47	30.30
36	11,090	2.77	28.40
48	4,800	1.96	14.40
72	3,179	3.10	6.00
Leucine From Blood γ-Globulins			
2	16,265	4.55	17.50
6	14,415	1.64	51.10
12	9,135	1.48	36.40
24	9,000	2.08	26.40
36	7,965	1.87	25.10
48	6,750	2.43	16.40
72	3,130	3.32	5.56
Leucine From Blood Albumin			
2	8,250	3.00	16.21
6	20,850	4.15	29.70
12	8,560	2.08	24.28
24	11,600	3.10	22.21
36	9,180	3.09	17.50
48	5,750	2.50	13.58
72	7,050	3.76	11.07
Leucine From "Blood" Serum Albumin From Milk Serum			
2	1,039	0.35	17.50
6	1,630	0.40	24.01
12	1,800	0.45	23.60
24	915	0.27	19.95
36	535	0.20	14.43
48	624	0.31	11.86
72	398	0.24	9.78

TABLE 4. (CONT.) SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-32, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 µC. OF LEUCINE-2-C^{14}

hrs. post inj.	DPM	mg. leucine counted	µc./mm. x 10^2
		Leucine From Blood α-Globulins	
2	1,194	4.48	1.58
6	4,300	1.29	19.70
12	5,125	1.90	15.90
24	2,950	1.85	9.40
72	1,290	3.63	2.09
		Leucine From Blood β-Globulins	
2	656	1.02	3.80
6	4,390	0.98	26.40
12	4,750	1.27	22.10
24	4,160	1.32	18.60
72	2,800	1.57	7.80

TABLE 5. RATIOS OF THE (a) SPECIFIC ACTIVITIES OF LEUCINE FROM MILK IMMUNE GLOBULINS TO THE (b) SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD γ-GLOBULINS FROM A LACTATING RABBIT (NO. X-32, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 μC. OF LEUCINE-2-C^{14}

hrs. post inj.	a/b	% of immune globulins derived from blood γ-globulins
2	1.040	100.0
6	0.948	94.8
12	1.058	100.0
24	1.140	100.0
36	1.130	100.0
48	0.878	87.8
72	1.078	100.0
	1.039 ± 0.117[1]	

[1] The mean value of the ratios is presented with standard error.

TABLE 6. RATIOS OF THE (a) SPECIFIC ACTIVITIES OF LEUCINE FROM "BLOOD" SERUM ALBUMIN FROM MILK TO THE (b) SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD ALBUMIN FROM A LACTATING RABBIT (NO. X-32, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 45.0 μC. OF LEUCINE-2-C^{14}

hrs. post inj.	a/b	% of milk "blood" serum albumin from milk serum derived from blood albumin
2	1.08	100.0
6	.81	81.0
12	.96	96.0
24	.90	90.0
36	.83	83.0
48	.875	87.5
72	.885	88.5
	0.906 ± 0.0367[1]	

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[1] The mean value of the ratios is presented with standard error.

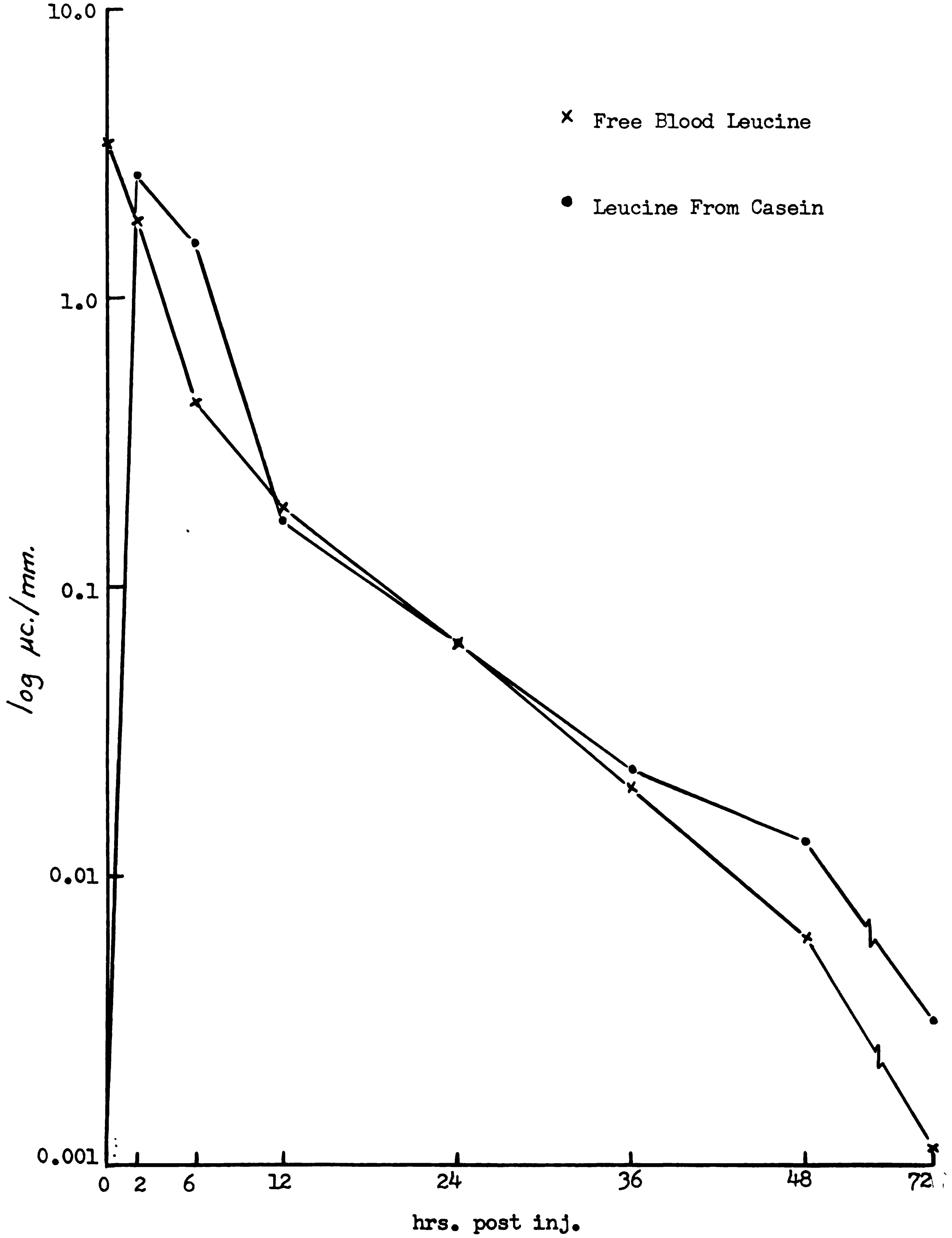

FIGURE 16. THE LOG OF SPECIFIC ACTIVITY (µc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM CASEIN ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 µC. OF DL-LEUCINE-2-C^{14}).

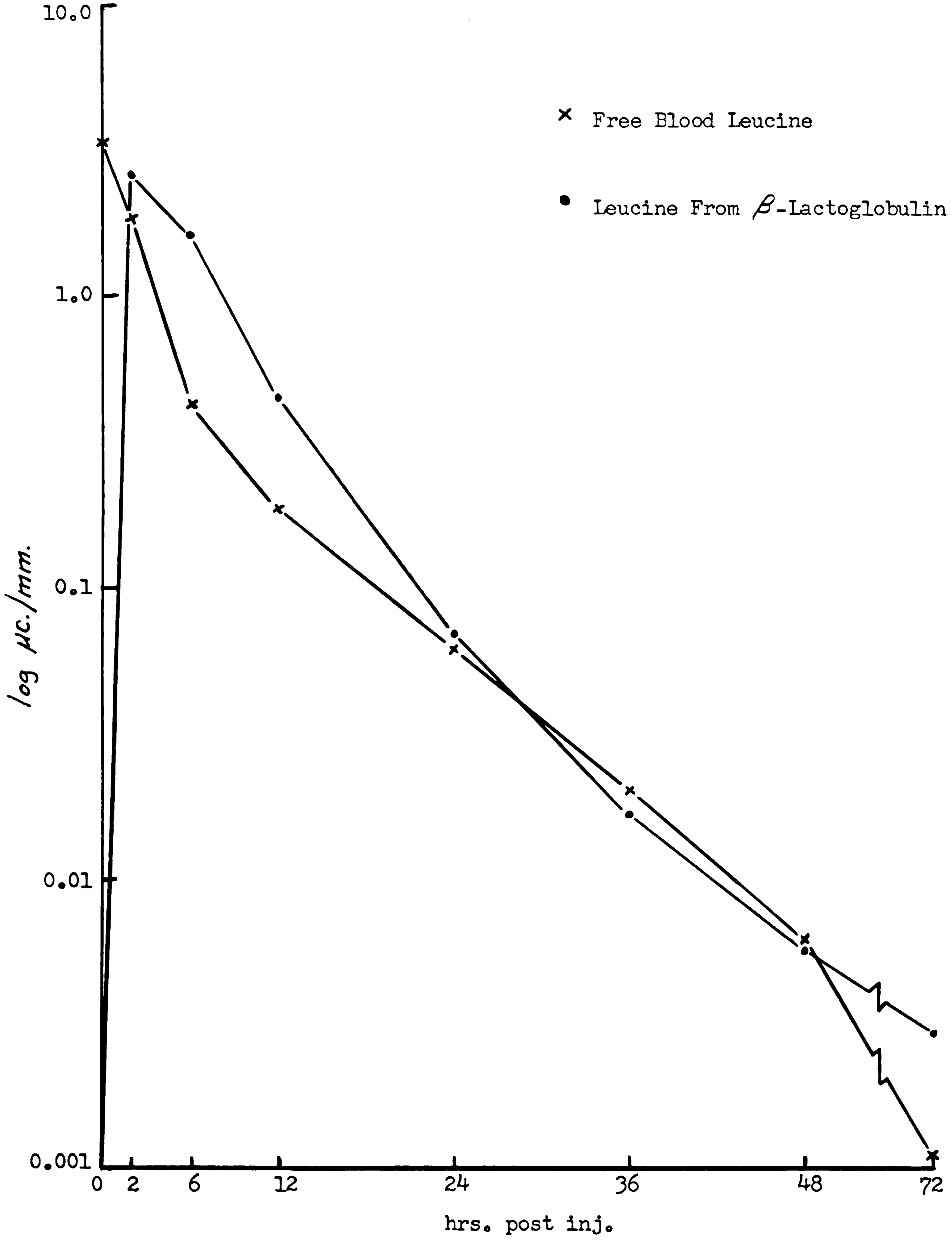

FIGURE 17. THE LOG OF SPECIFIC ACTIVITY (µc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM β-LACTOGLOBULIN ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 µC. OF DL-LEUCINE-2-C^{14}).

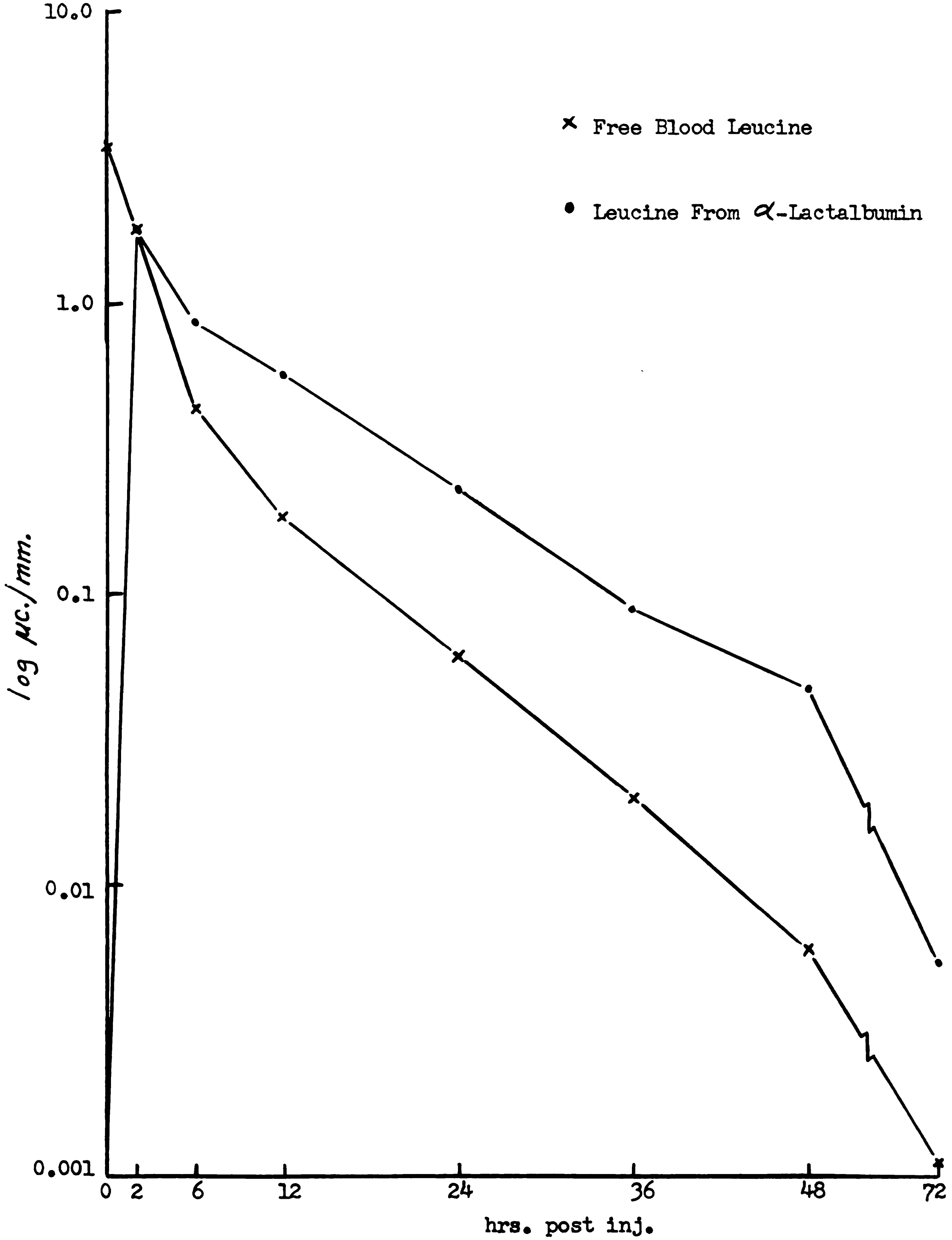

FIGURE 18. THE LOG OF SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM α-LACTALBUMIN ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

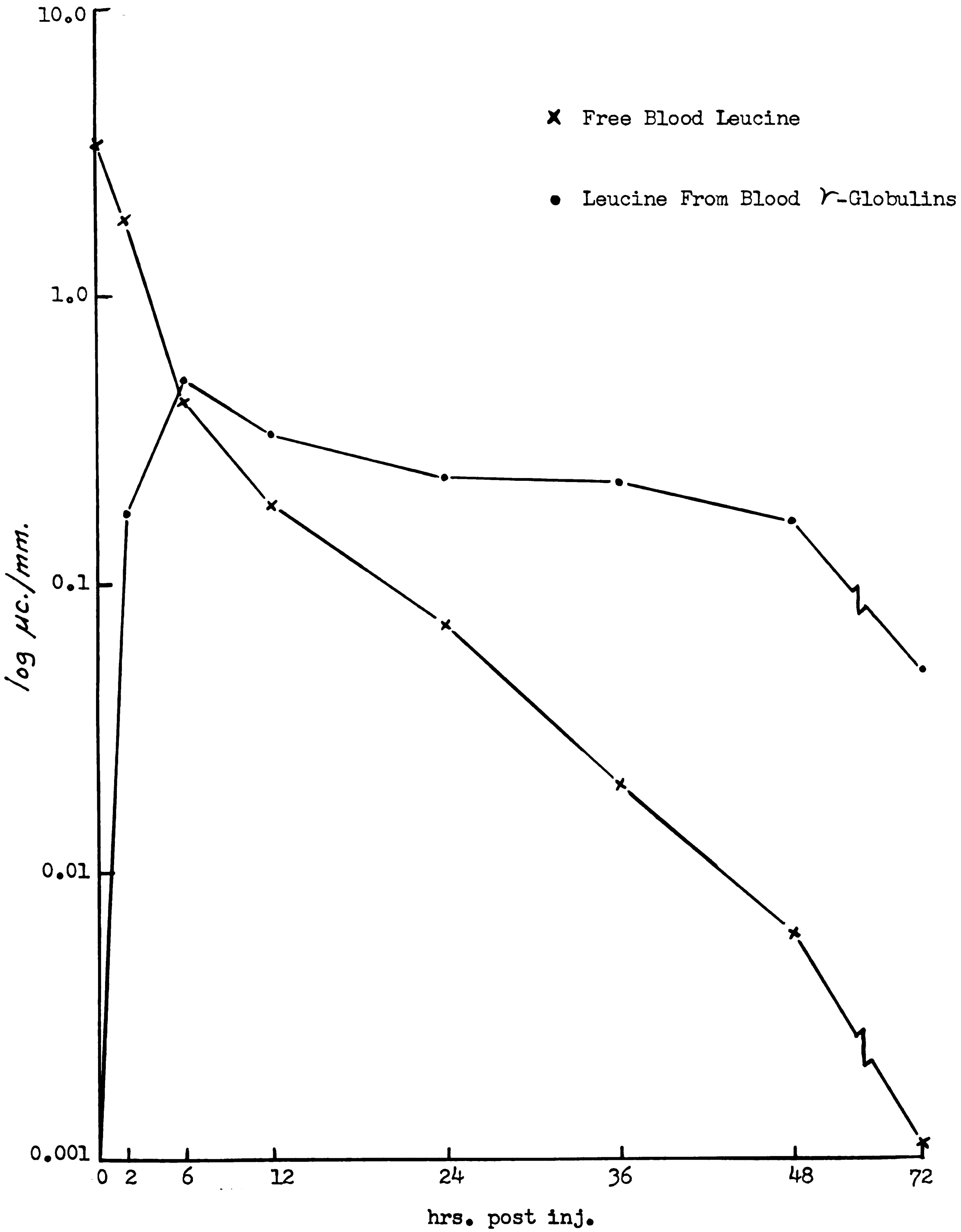

FIGURE 19. THE LOG OF SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM BLOOD γ-GLOBULINS ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. DL-LEUCINE-2-C^{14}).

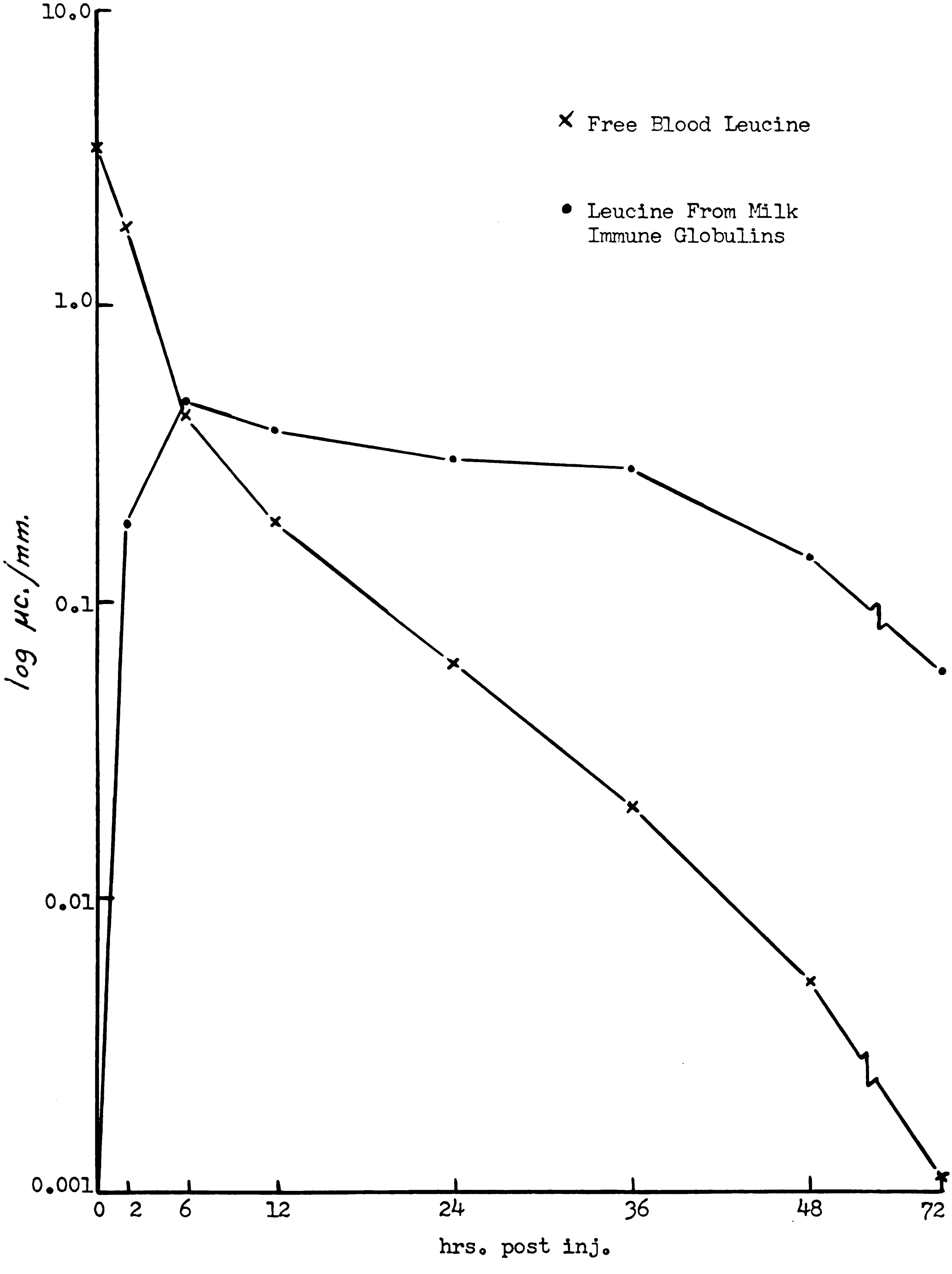

FIGURE 20. THE LOG OF SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM MILK IMMUNE GLOBULINS ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

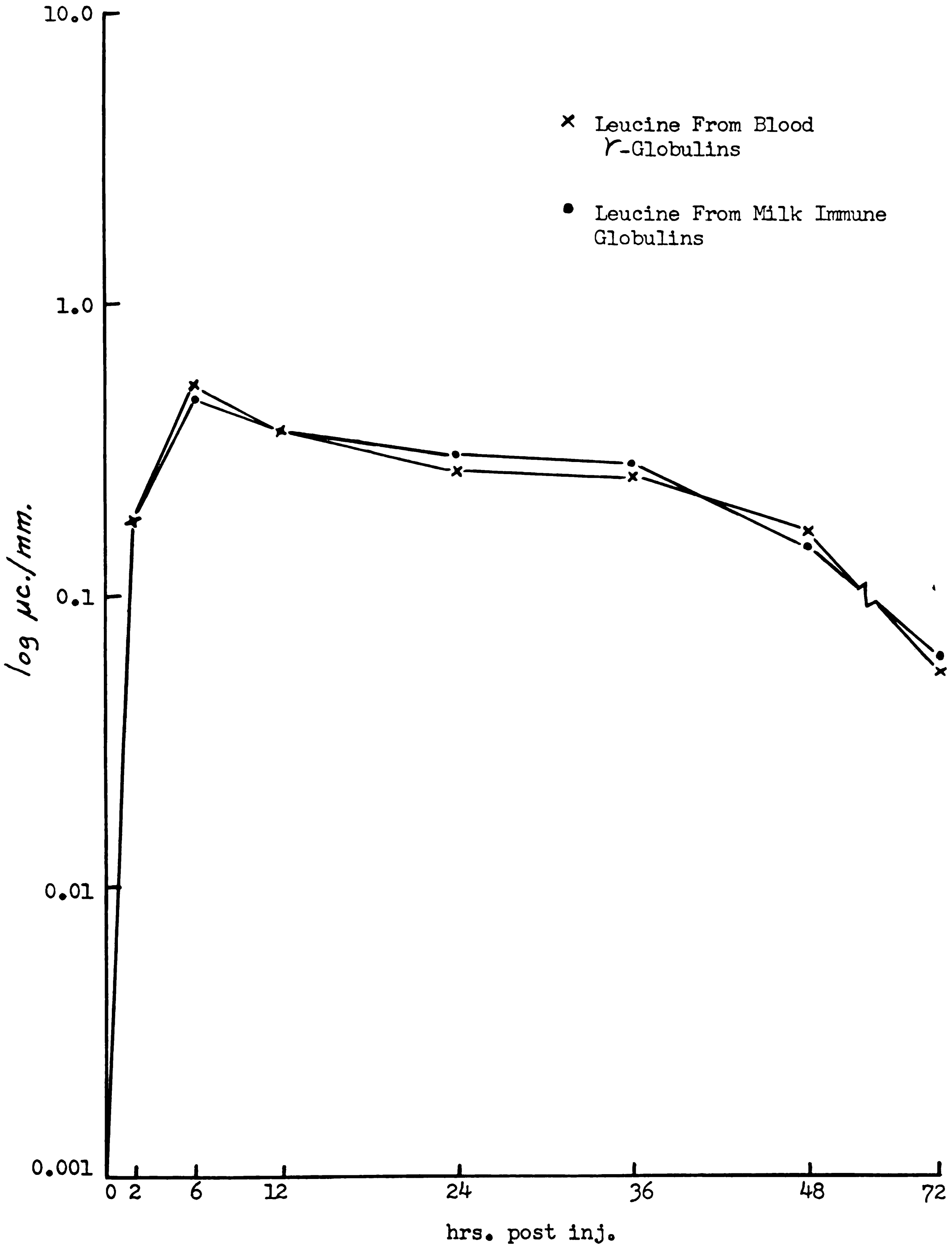

FIGURE 21. THE LOG OF SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR LEUCINE FROM BLOOD γ-GLOBULINS AND MILK IMMUNE GLOBULINS ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. DL-LEUCINE-2-C^{14}).

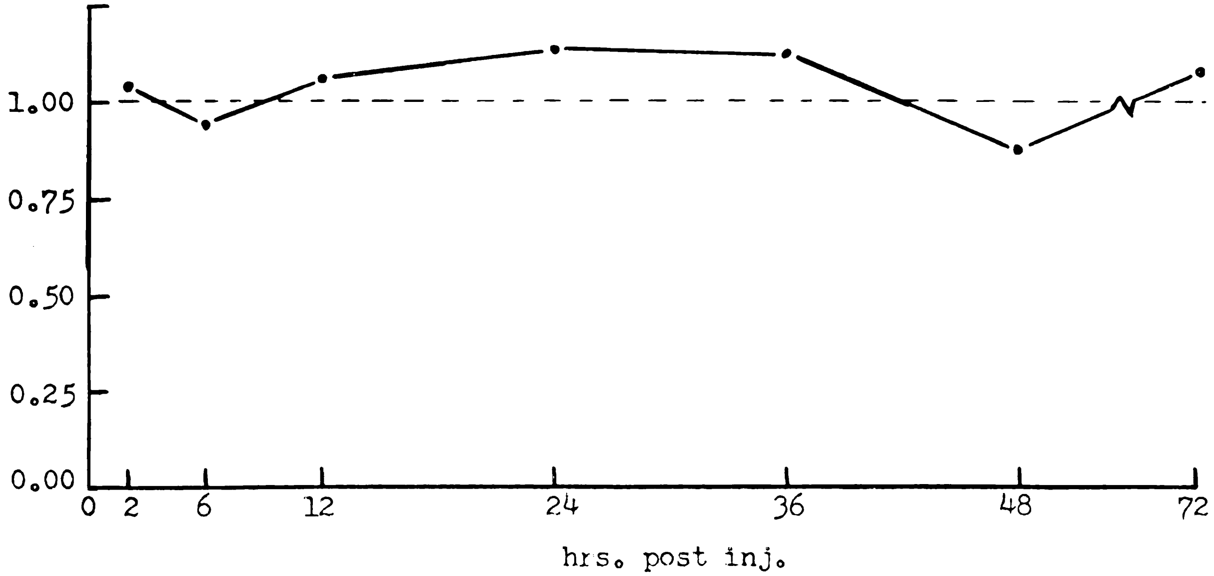

FIGURE 22. RATIOS OF THE SPECIFIC ACTIVITIES OF LEUCINE FROM MILK IMMUNE GLOBULINS TO THE SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD γ-GLOBULINS ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

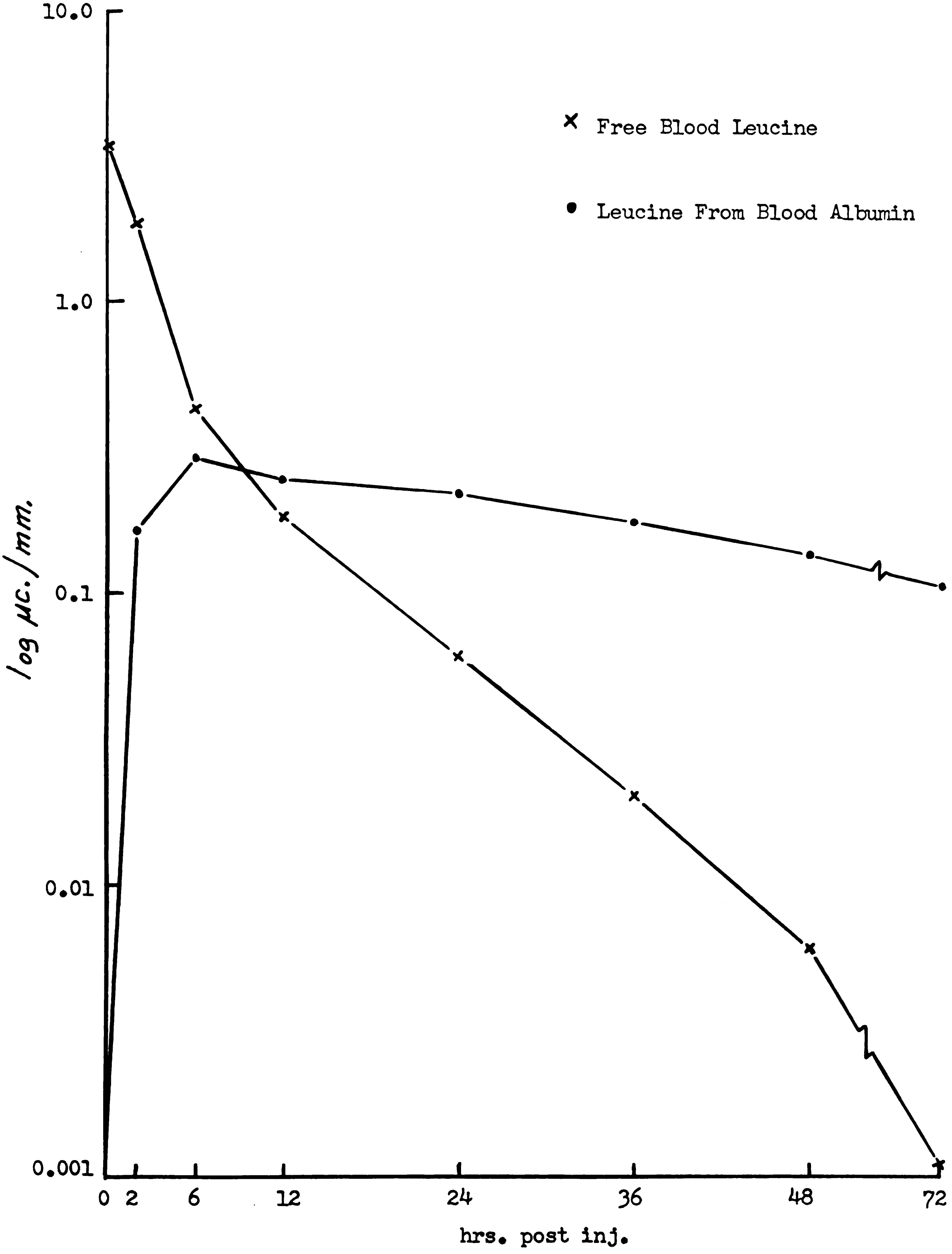

FIGURE 23. THE LOG OF SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM BLOOD ALBUMIN ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

log μc./mm.

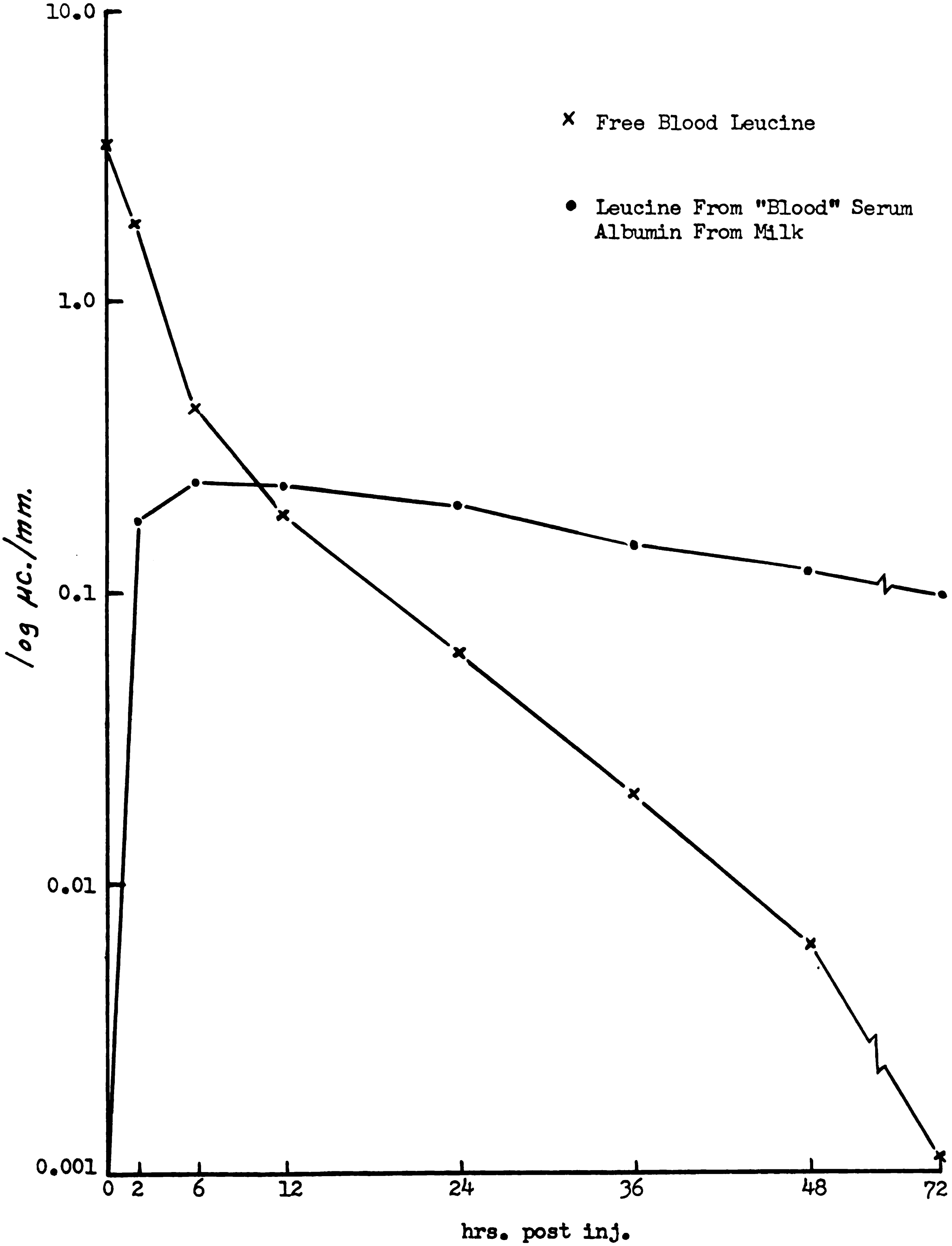

FIGURE 24. THE LOG OF THE SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. DL-LEUCINE-2-C^{14}).

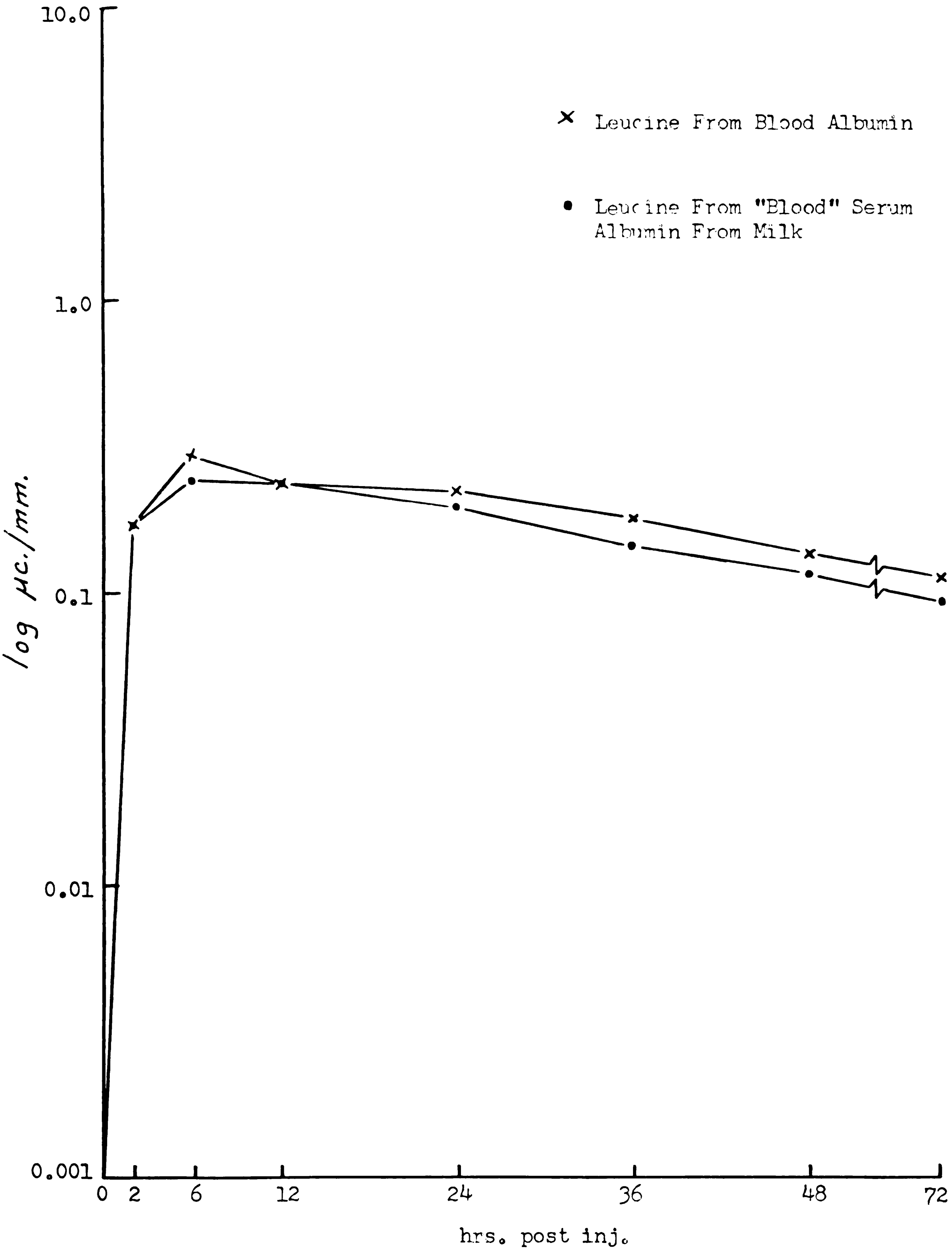

FIGURE 25. THE LOG OF SPECIFIC ACTIVITY (µc./mm.) VS. TIME (hrs. post inj.) FOR LEUCINE FROM BLOOD ALBUMIN AND "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POST-PARTUM INJECTED WITH 45.0 µC. OF DL-LEUCINE-2-C^{14}).

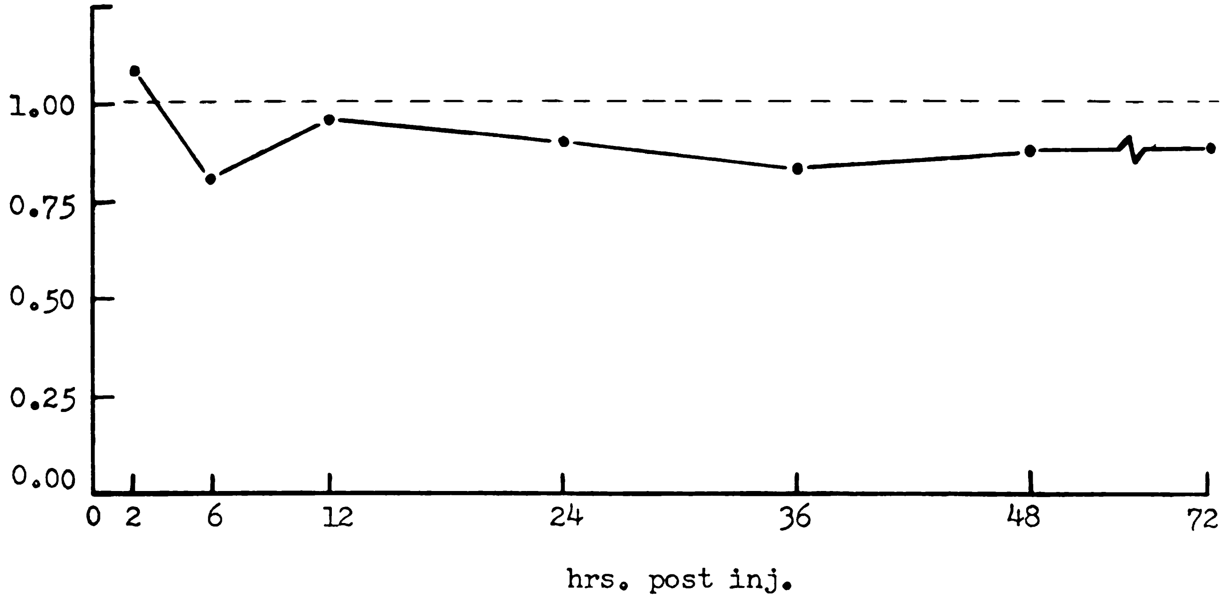

FIGURE 26. RATIOS OF THE SPECIFIC ACTIVITIES OF LEUCINE FROM "BLOOD" SERUM ALBUMIN FROM MILK TO THE SPECIFIC ACTIVITIES OF LEUCINE FROM BLOOD ALBUMIN ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. OF DL-LEUCINE-2-C^{14}).

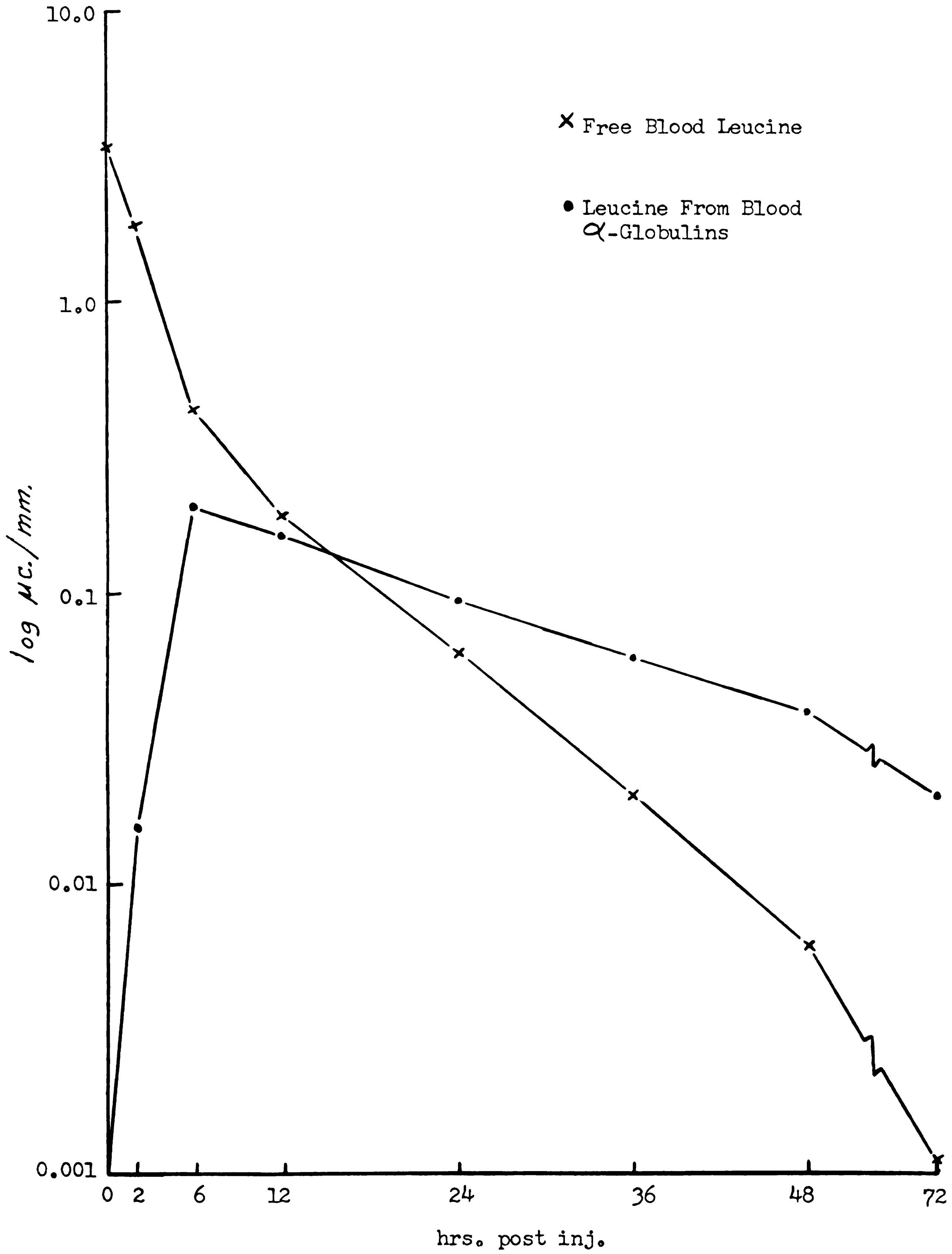

FIGURE 27. THE LOG OF SPECIFIC ACTIVITY (μc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM BLOOD α-GLOBULINS ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 μC. DL-LEUCINE-2-C^{14}).

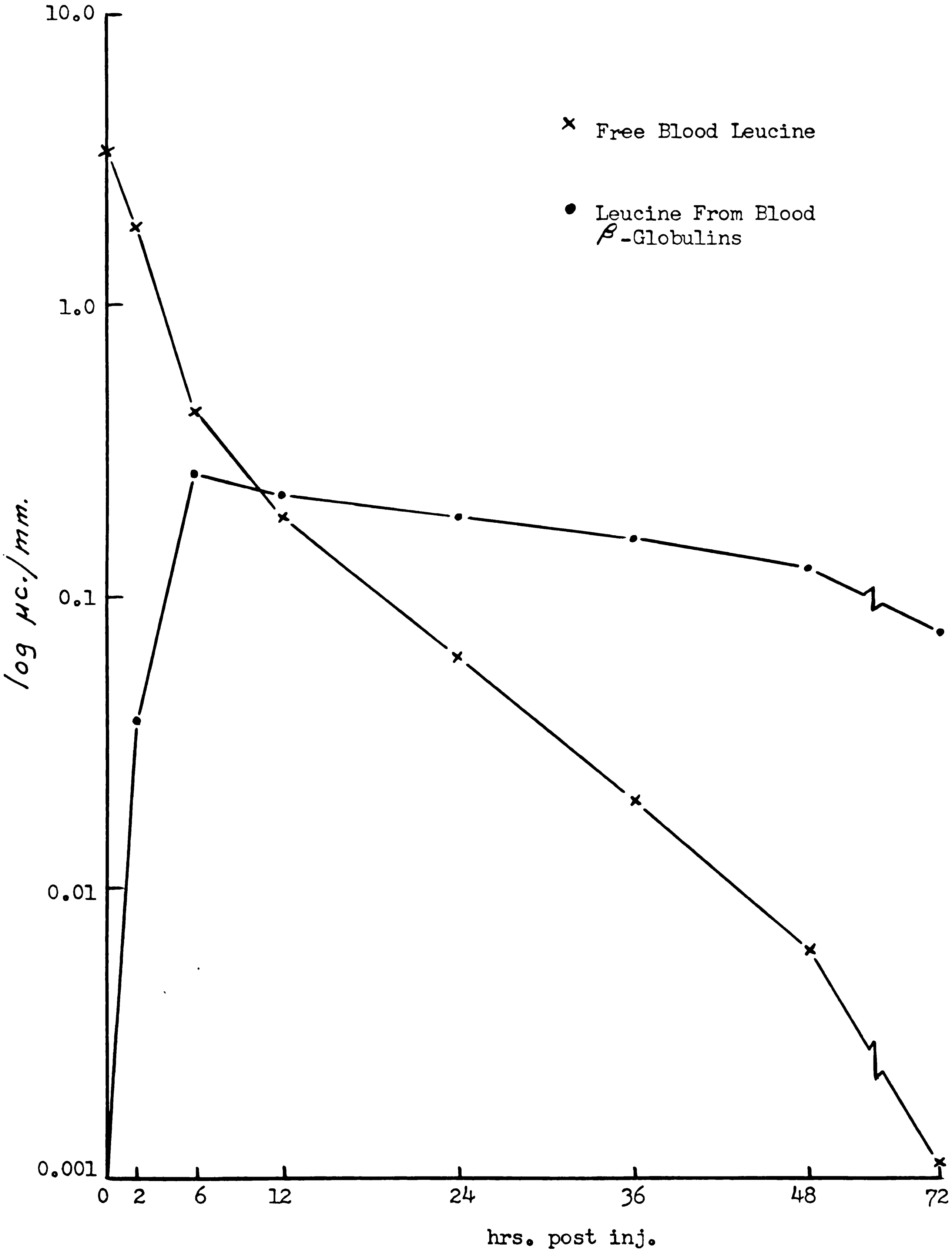

FIGURE 28. THE LOG OF THE SPECIFIC ACTIVITY (µc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD LEUCINE AND LEUCINE FROM β-GLOBULINS ISOLATED FROM X-32 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 µC. OF DL-LEUCINE-2-C^{14}).

TABLE 7. SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-30, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 μC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	DPM	mg. glutamic acid counted	μc./mm. x 10
		Free Blood Glutamic Acid	
0.16	35,700	0.100	250.00
2	8,137	0.070	76.80
6	4,019	0.050	53.20
12	1,411	0.047	19.80
24	528	0.049	7.10
36	252	0.050	3.30
48	102	0.044	1.50
72	61	0.100	0.40
		Glutamic Acid From Casein	
2	152,150	1.100	91.60
6	126,900	1.000	84.00
12	660,500	11.100	39.10
24	257,000	10.400	16.40
36	152,200	10.000	10.80
48	48,390	9.700	3.29
72	27,695	10.100	1.81
		Glutamic Acid From Milk β-Lactoglobulin	
2	158,500	1.00	105.00
6	122,500	1.03	78.90
12	96,500	1.10	58.10
24	308,000	8.50	24.00
36	127,000	9.70	8.60
48	69,000	10.00	4.56
72	18,300	6.60	1.21
		Glutamic Acid From Milk α-Lactalbumin	
2	645,000	7.80	54.75
6	585,000	8.40	46.10
12	453,000	8.10	37.00
24	246,000	7.90	23.10
36	102,500	7.80	8.72
48	36,900	7.70	3.18
72	13,470	7.80	1.14

TABLE 7. (CONT.) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-30, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 μC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	DPM	mg. glutamic acid counted	μc./mm. x 10
	Glutamic Acid From Milk Immune Globulins		
2	9,140	5.40	1.12
6	22,485	4.90	3.04[1]
12	18,800	4.70	2.65
24	14,250	5.00	1.89
36	8,610	4.10	1.39
48	6,715	4.40	1.01
72	3,380	2.90	0.77
	Glutamic Acid From Blood γ-Globulins		
2	9,165	4.70	1.29
6	12,400	2.60	2.92
12	8,480	2.00	2.80
24	6,780	2.30	1.95
36	7,815	3.50	1.48
48	6,495	3.90	1.10
72	7,850	6.20	0.84
	Glutamic Acid From Blood Albumin		
2	23,900	8.30	1.89
6	36,900	7.90	3.09
12	25,800	6.80	2.51
24	22,400	8.50	1.74
36	12,600	8.00	1.04
48	9,960	7.20	0.92
72	5,200	6.90	0.50
	Glutamic Acid From "Blood" Serum Albumin Fraction From Milk Serum		
2	63	0.030	1.39
6	107	0.270	2.62
12	71	0.020	2.34
24	66	0.025	1.74
36	34	0.023	0.99
48	20[2]	0.020	0.66
72	45	0.060	0.50

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[1] This value is not significantly different from the specific activity of blood γ-globulins at 6 hours.

[2] The count of this sample was not significantly different from background.

TABLE 7. (CONT.) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-30, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 µC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	DPM	mg. glutamic acid counted	µc/mm. x 10
	Glutamic Acid From Blood α-Globulins		
2	12,500	4.70	1.76
6	35,390	8.30	2.72
12	17,590	3.70	3.15
24	32,190	9.60	2.10
36	14,100	5.00	1.87
48	5,290	4.50	0.80
72	6,690	7.70	0.57
	Glutamic Acid From Blood β-Globulins		
2	24,400	4.70	3.44
6	23,000	2.60	5.85
12	17,830	4.10	2.88
24	10,710	4.20	1.69
36	8,740	4.50	1.27
48	5,110	3.30	1.02
72	4,670	6.20	0.50

TABLE 8. RATIOS OF THE (a) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM MILK IMMUNE GLOBULINS TO THE (b) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD γ-GLOBULINS FROM A LACTATING RABBIT (NO. X-30, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 μC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	a/b	% of immune globulins derived from blood γ-globulins
2	0.868	86.8
6	1.040	100.0
12	0.948	94.8
24	0.970	97.0
36	0.939	93.9
48	0.918	91.8
72	0.922	92.2
	0.943 ± 0.0141[1]	

[1] The mean of the ratios is presented with standard error.

TABLE 9. RATIOS OF THE (a) SPECIFIC ACTIVITIES OF "BLOOD" SERUM ALBUMIN FROM MILK SERUM TO THE (b) SPECIFIC ACTIVITIES OF BLOOD ALBUMIN FROM A LACTATING RABBIT (NO. X-30, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 μC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	a/b	% of "blood" serum albumin from milk serum derived from blood serum albumin
2	0.735	73.5
6	0.850	85.0
12	0.933	93.3
24	0.998	99.8
36	0.948	94.8
48	0.722	72.2
72	1.000	100.0
	0.884 ± 0.0386[1]	

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[1] The mean of the ratios is presented with standard error.

TABLE 10. SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-34, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 µC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	DPM	mg. free glutamic acid counted	µc./mg. x 10
		Free Blood Glutamic Acid	
0.16	35,200	0.110	209.000
2	14,050	0.090	103.400
6	5,560	0.078	47.300
12	1,950	0.049	26.350
24	648	0.040	10.700
36	501	0.055	3.320
48	131	0.046	1.890
72	159	0.130	0.809
		Glutamic Acid From Casein	
2	270,500	1.500	119.000
6	131,300	1.300	66.800
12	606,500	10.500	38.200
24	231,500	9.800	15.600
36	87,550	8.600	6.730
48	56,900	10.200	3.690
72	19,490	10.900	1.180
		Glutamic Acid From Milk β-Lactoglobulin	
2	151,100	1.100	91.000
6	248,000	2.500	65.700
12	373,000	5.300	46.600
24	392,000	9.800	26.500
36	151,100	10.500	9.950
48	62,650	7.600	5.550
72	24,800	4.900	3.350
		Glutamic Acid From Milk α-Lactalbumin	
2	668,000	8.900	49.700
6	391,900	5.700	45.500
12	396,000	7.300	35.900
24	165,800	6.800	16.100
36	85,900	9.100	6.250
48	38,350	6.500	3.910
72	19,550	8.400	1.540

TABLE 10. (CONT.) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-34, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 µC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	DPM	mg. free glutamic acid counted	µc./mg. x 10
	Glutamic Acid From Milk Immune Globulins		
2	13,100	6.100	1.425
6	20,300	4.700	2.860
12	20,600	5.000	2.730
24	10,180	3.300	2.010
36	7,400	2.900	1.690
48	7,870	4.200	1.240
72	5,765	5.800	0.659
	Glutamic Acid From Blood γ-Globulins		
2	11,990	5.100	1.550
6	16,000	3.500	3.020
12	12,700	2.900	2.900
24	13,680	4.000	2.260
36	7,230	2.600	1.840
48	6,560	3.300	1.317
72	7,840	6.700	0.775
	Glutamic Acid From Blood Albumin		
2	20,690	8.000	1.710
6	24,850	5.900	2.790
12	21,400	6.300	2.250
24	16,410	6.800	1.580
36	11,200	7.500	0.990
48	10,780	8.600	0.830
72	7,210	8.100	0.590
	Glutamic Acid From "Blood" Albumin From Milk Serum		
2	62.7	0.029	1.430
6	122.1	0.031	2.610
12	63.2	0.020	2.090
24	37.9	0.017	1.480
36	35.9	0.025	0.950
48	21.5[1]	0.018	0.790
72	46.1	0.050	0.610

---

[1] The count of this sample was not significantly different from background.

TABLE 10. (CONT.) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-34, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 μC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	DPM	mg. free glutamic acid counted	μc./mg. x 10
Glutamic Acid From Blood α-Globulins			
2	22,200	9.200	1.600
6	29,520	7.700	2.540
12	23,570	5.400	2.890
24	18,500	4.900	2.500
36	20,750	6.300	2.180
48	23,580	7.800	2.000
72	19,740	8.900	1.470
Glutamic Acid From Blood β-Globulins			
2	28,750	6.400	2.750
6	34,570	4.900	4.670
12	20,380	3.200	4.210
24	25,350	5.500	3.050
36	15,230	5.800	1.740
48	10,680	6.100	1.160
72	4,560	6.300	0.480

TABLE 11. RATIOS OF THE (a) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM MILK IMMUNE GLOBULINS TO THE (b) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD γ-GLOBULINS FROM A LACTATING RABBIT (NO. X-34, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 μC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	a/b	% of milk immune globulins derived from blood γ-globulins
2	0.917	91.7
6	0.948	94.8
12	0.943	94.3
24	0.889	88.9
36	0.918	91.8
48	0.944	94.4
72	0.850	85.0
	0.916 ± 0.0135[1]	

[1] The mean value for the ratios is presented with standard error.

TABLE 12. RATIOS OF THE (a) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM "BLOOD" SERUM ALBUMIN FROM MILK TO THE (b) SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD ALBUMIN FROM A LACTATING RABBIT (NO. X-34, 14TH-17TH DAYS POSTPARTUM) INJECTED WITH 150 μC. OF GLUTAMIC ACID-2-C^{14}

hrs. post inj.	a/b	% of "blood" serum albumin from milk serum derived from blood albumin
2	0.836	83.6
6	0.935	93.5
12	0.929	92.9
24	0.937	93.7
36	0.960	96.0
48	0.952	95.2
72	1.033	103.3
	0.940 ± 0.0216[1]	

[1] The mean value of the ratios is presented with standard error.

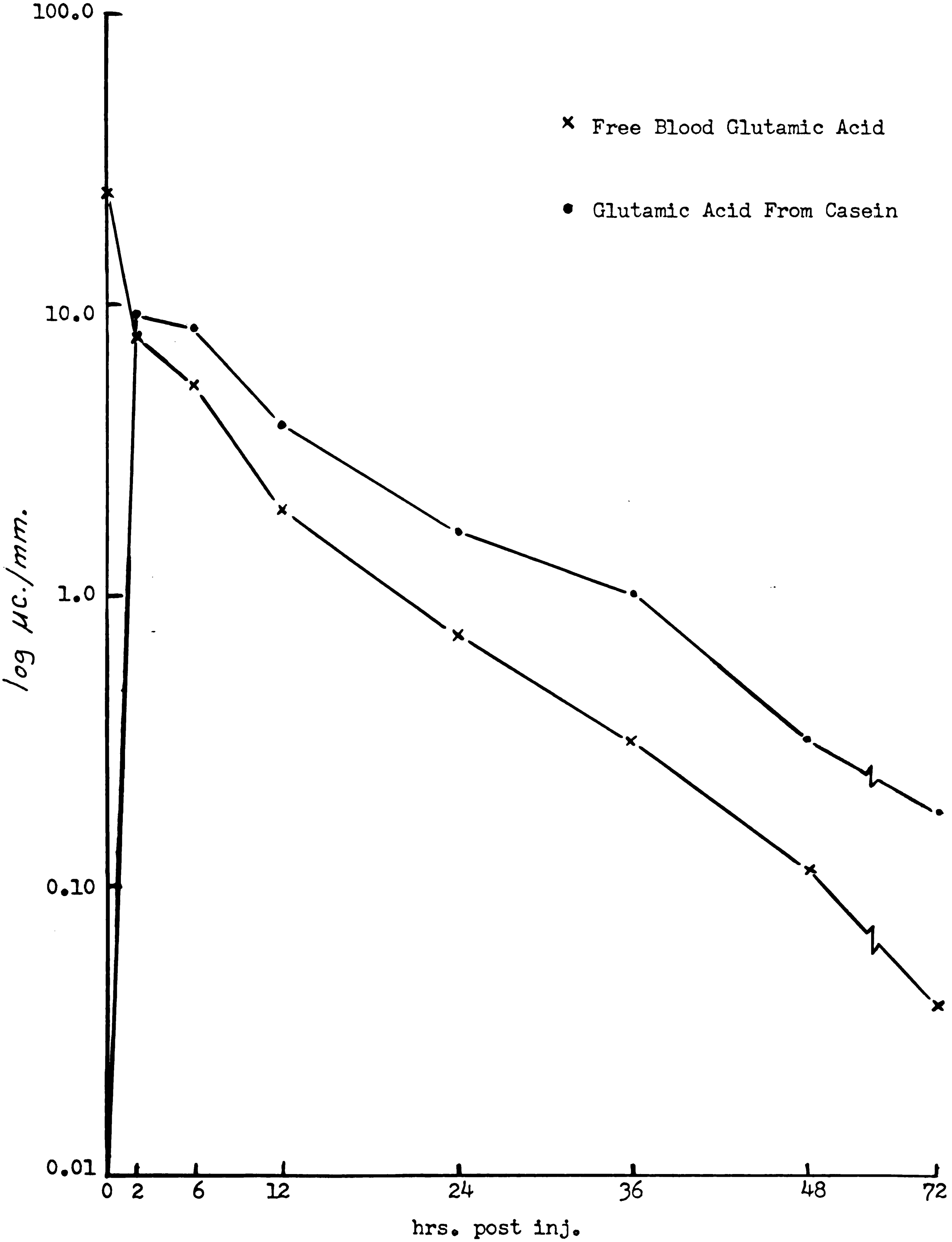

FIGURE 29. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM CASEIN ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 µC. OF DL-GLUTAMIC ACID-2-C^{14}).

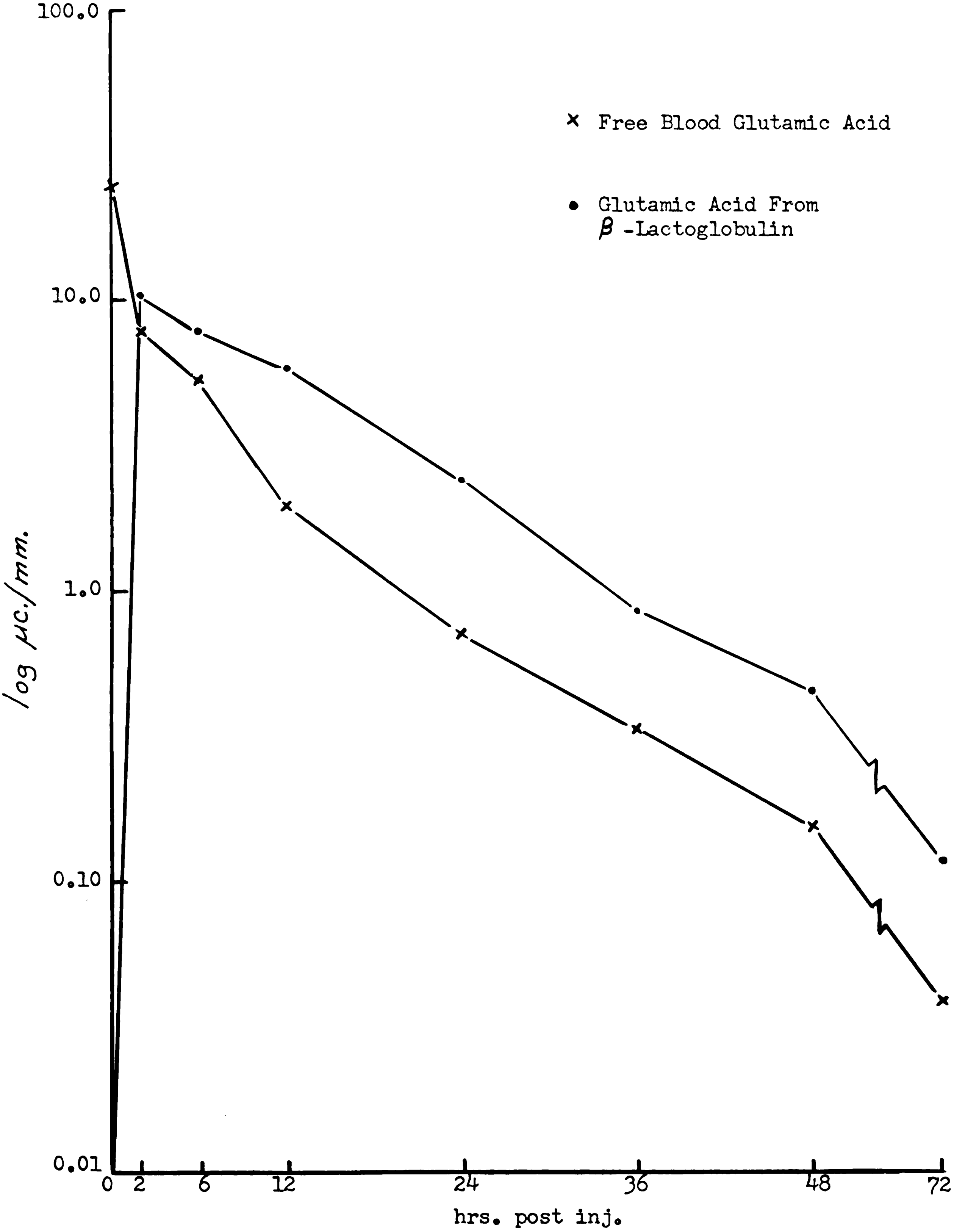

FIGURE 30. THE LOG OF THE SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM β-LACTOGLOBULIN ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 µC. OF DL-GLUTAMIC ACID-2-C^{14}).

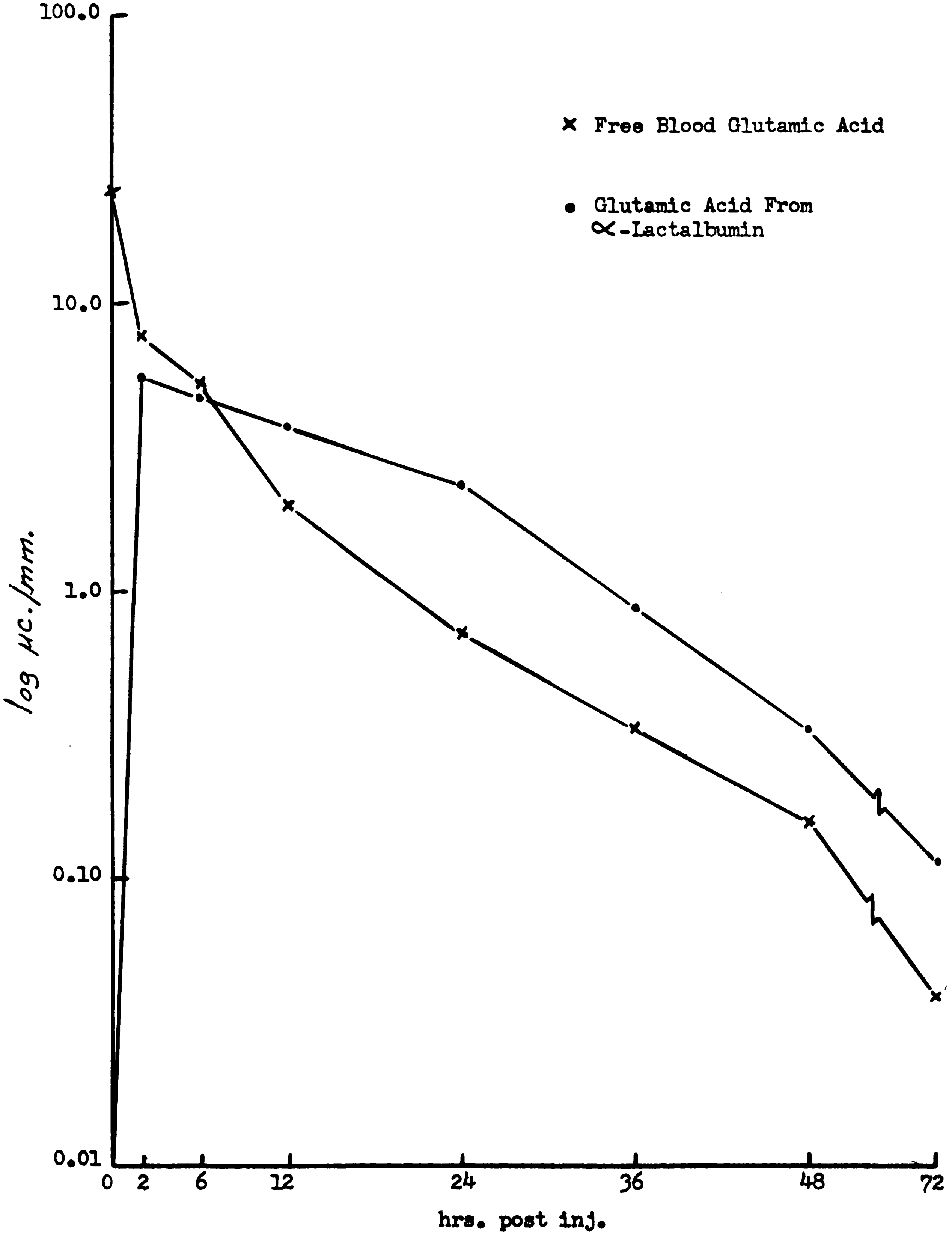

FIGURE 31. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM ∝-LACTALBUMIN ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

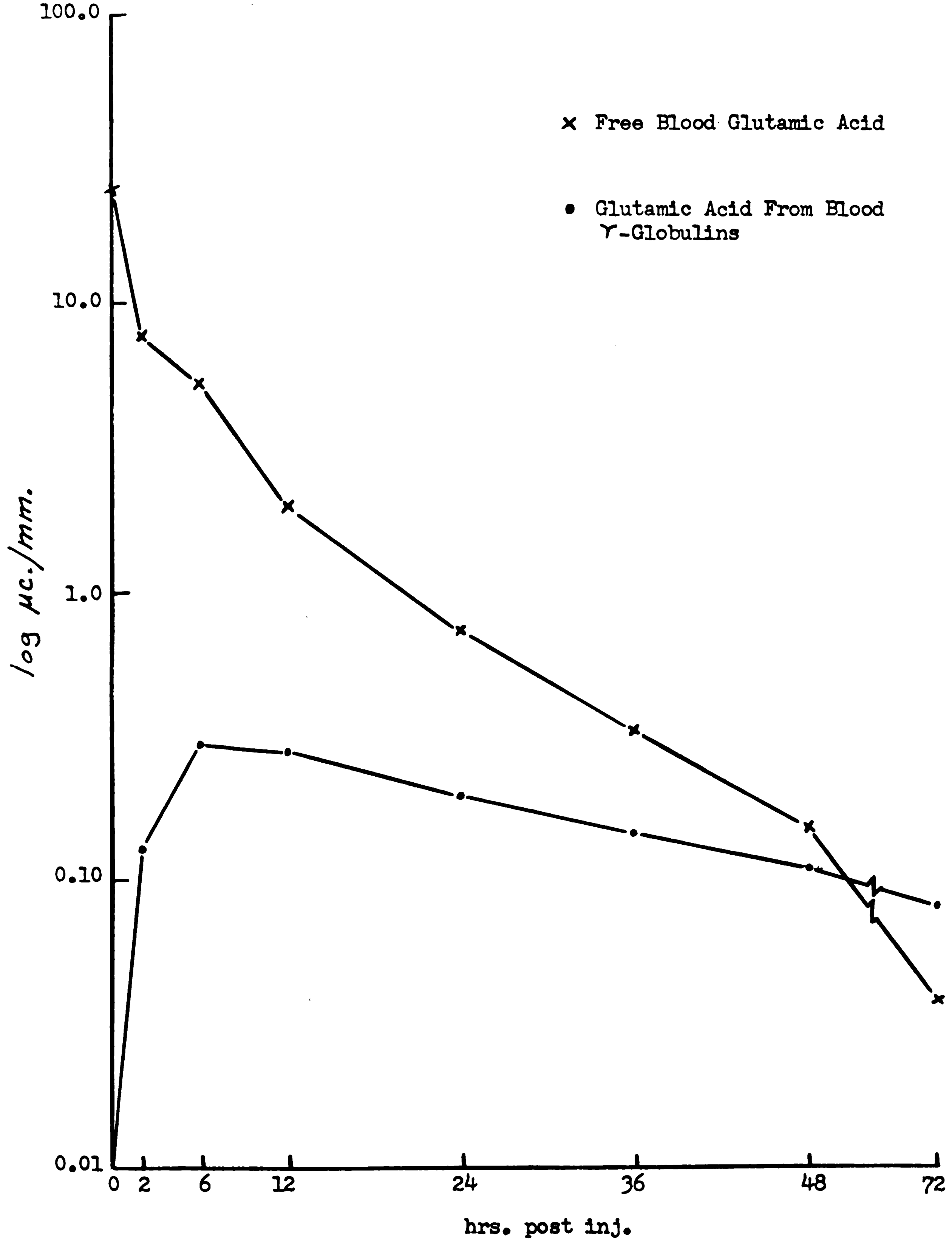

FIGURE 32. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM BLOOD γ-GLOBULINS ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 µC. OF DL-GLUTAMIC ACID-2-C^{14}).

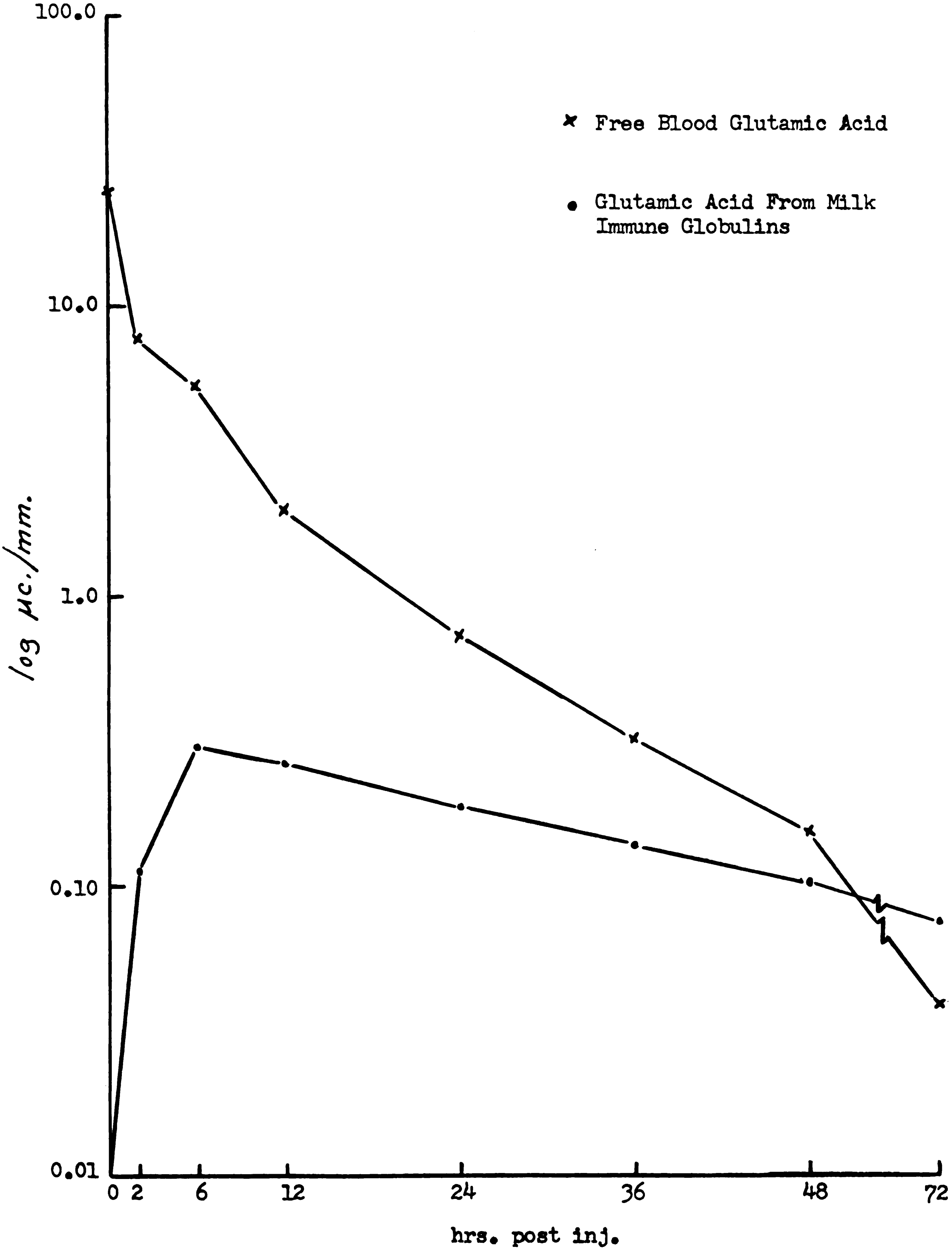

FIGURE 33. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post. inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM MILK IMMUNE GLOBULINS ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

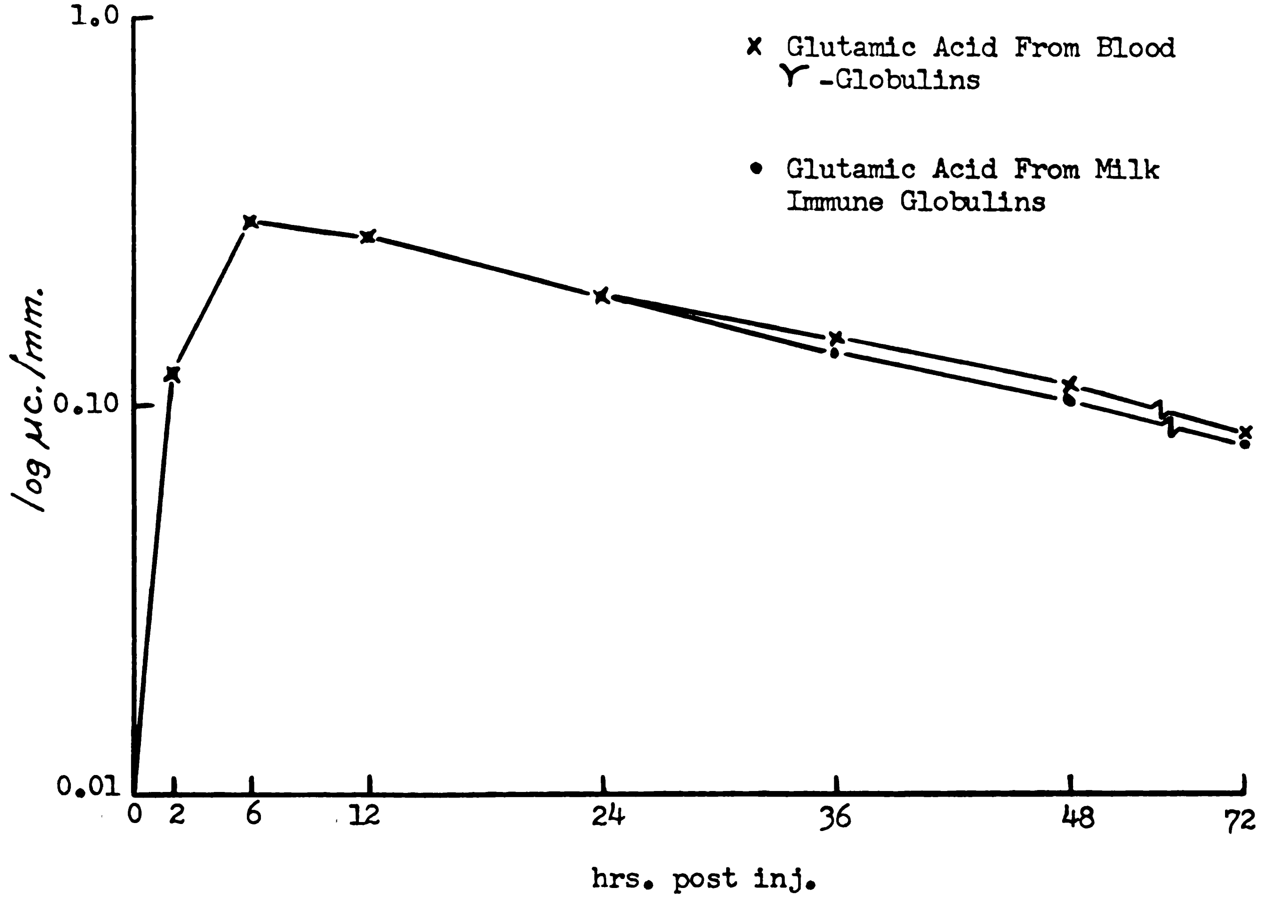

FIGURE 34. THE LOG OF THE SPECIFIC ACTIVITY (uc./mm.) OF GLUTAMIC ACID FROM BLOOD γ-GLOBULINS AND MILK IMMUNE GLOBULINS ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

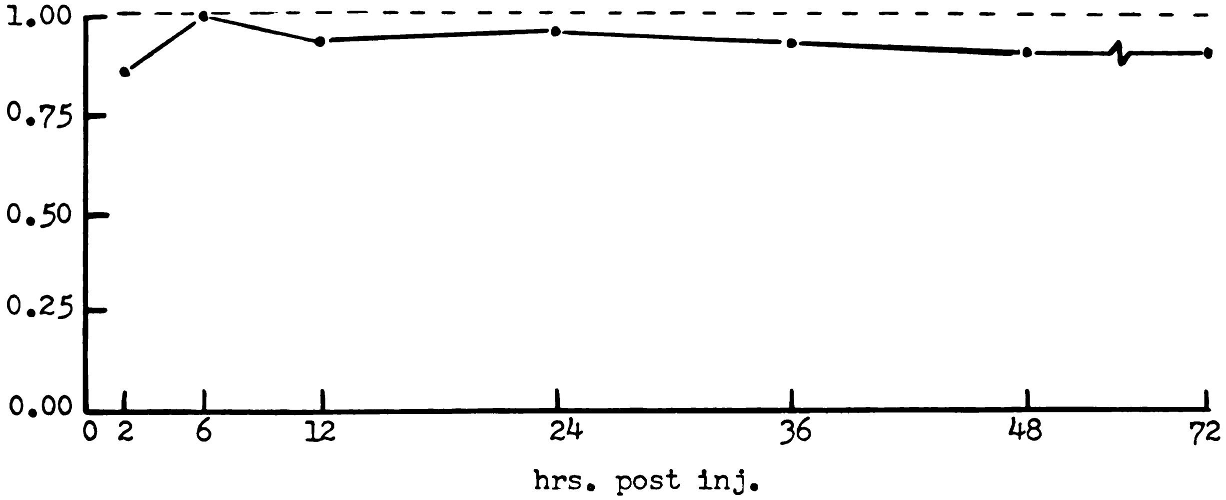

FIGURE 35. RATIOS OF THE SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM MILK IMMUNE GLOBULINS TO THE SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD γ-GLOBULINS FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150 μC. DL-GLUTAMIC ACID-2-C^{14}).

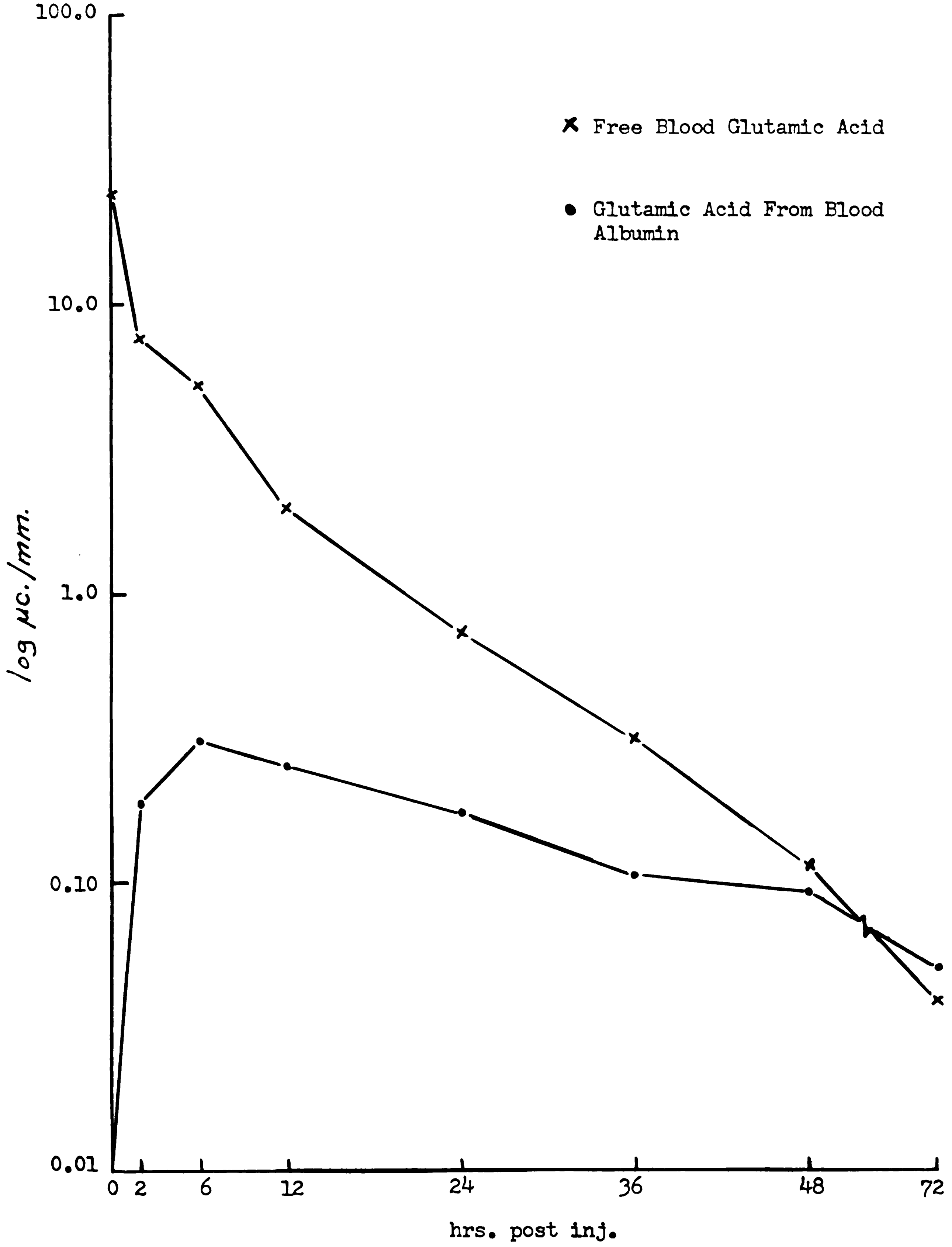

FIGURE 36. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM BLOOD ALBUMIN ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

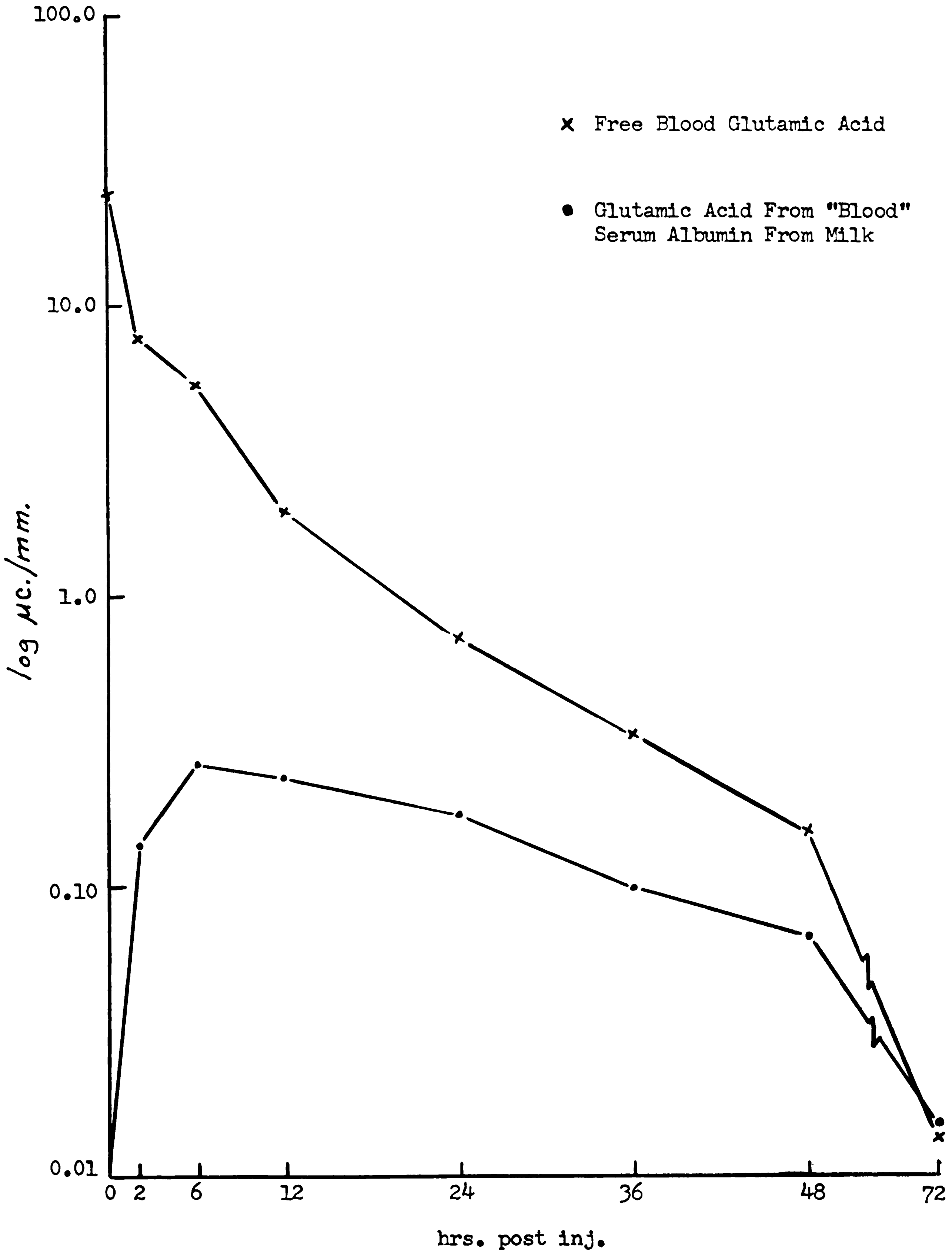

FIGURE 37. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 µC. OF DL-GLUTAMIC ACID-2-C^{14}).

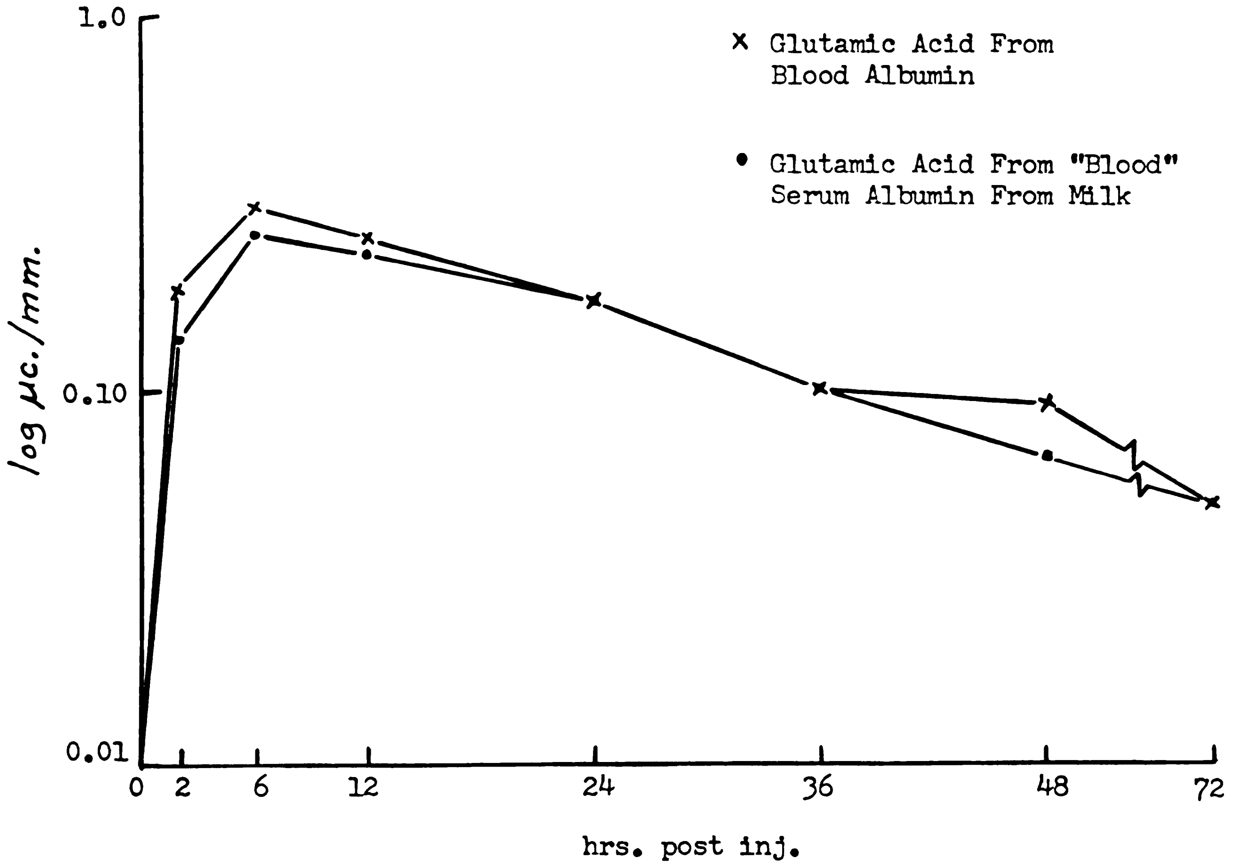

FIGURE 38. THE LOG OF THE SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR GLUTAMIC ACID FROM BLOOD ALBUMIN AND "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

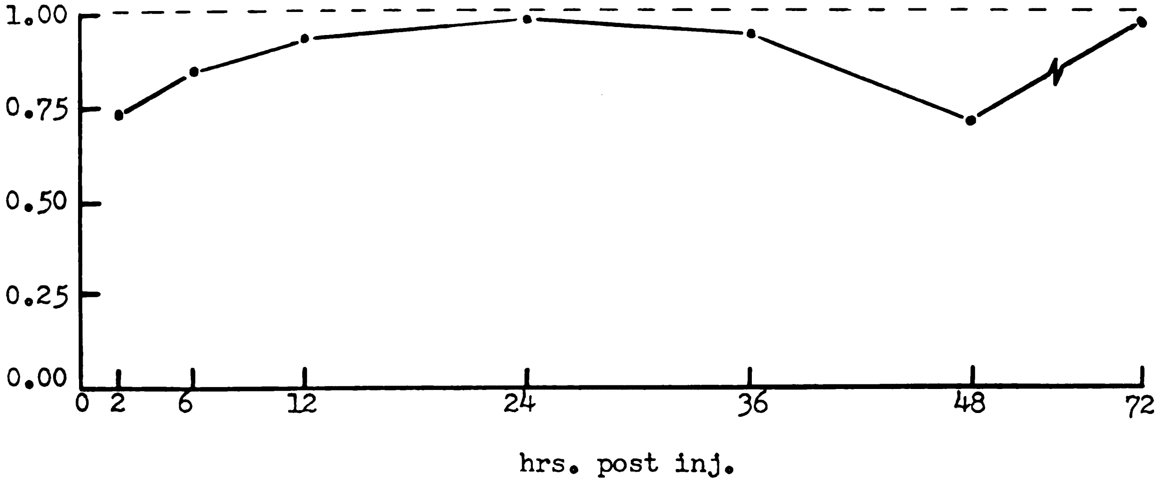

FIGURE 39. RATIOS OF THE SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM "BLOOD" SERUM ALBUMIN FROM MILK TO THE SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD ALBUMIN ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

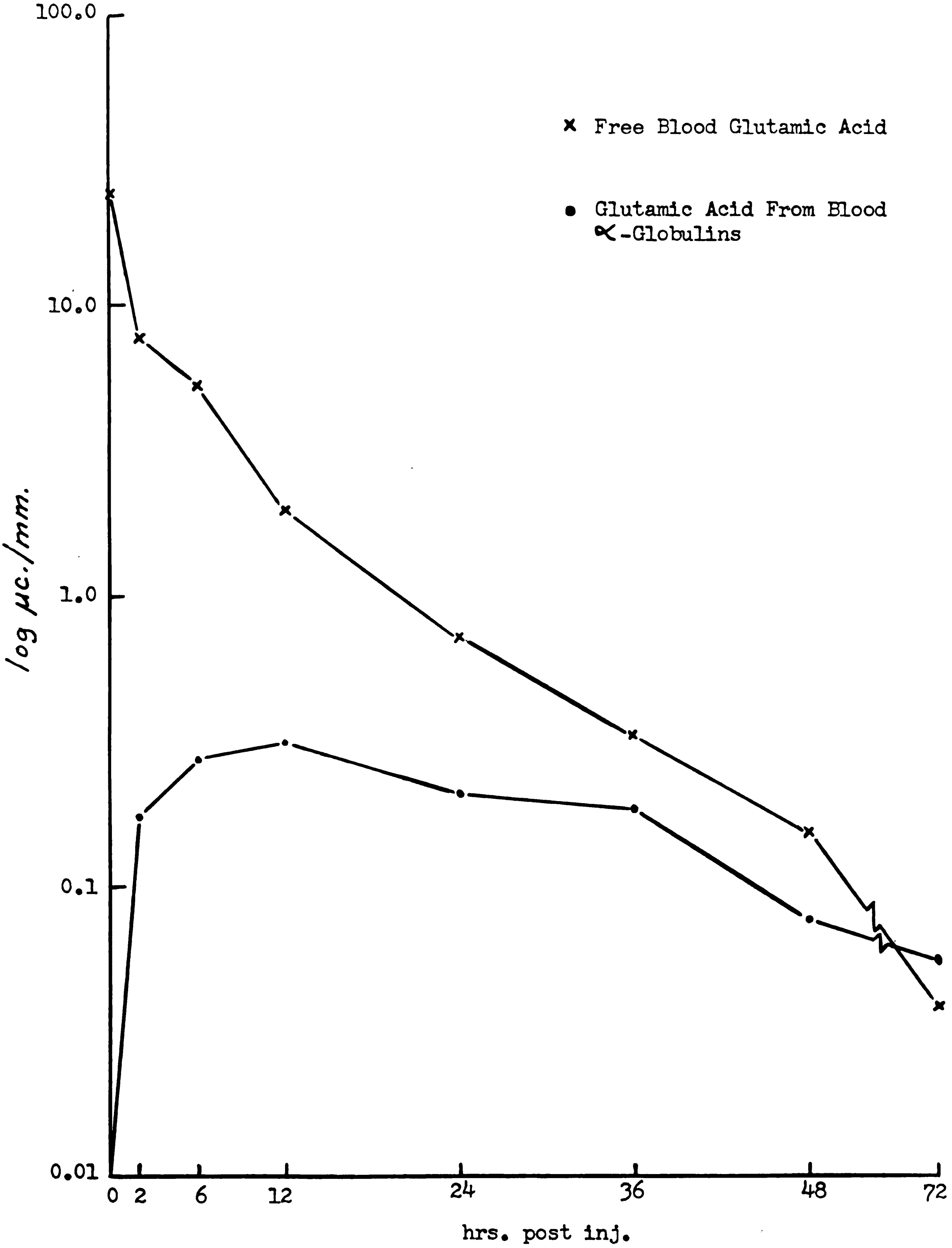

FIGURE 40. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM BLOOD ∝-GLOBULINS ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

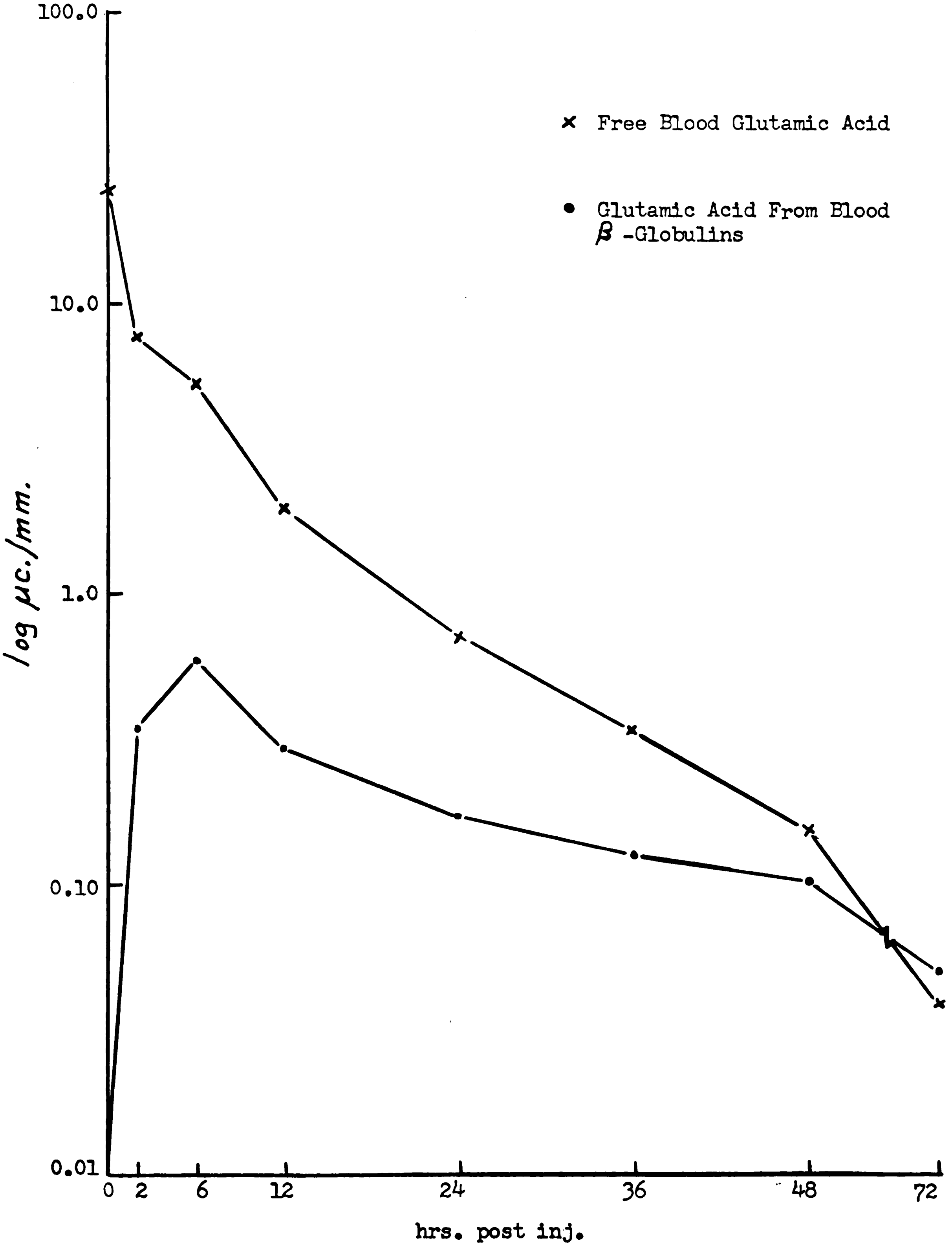

FIGURE 41. LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM BLOOD β -GLOBULINS ISOLATED FROM X-30 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 µC. OF DL-GLUTAMIC ACID-2-C^{14}).

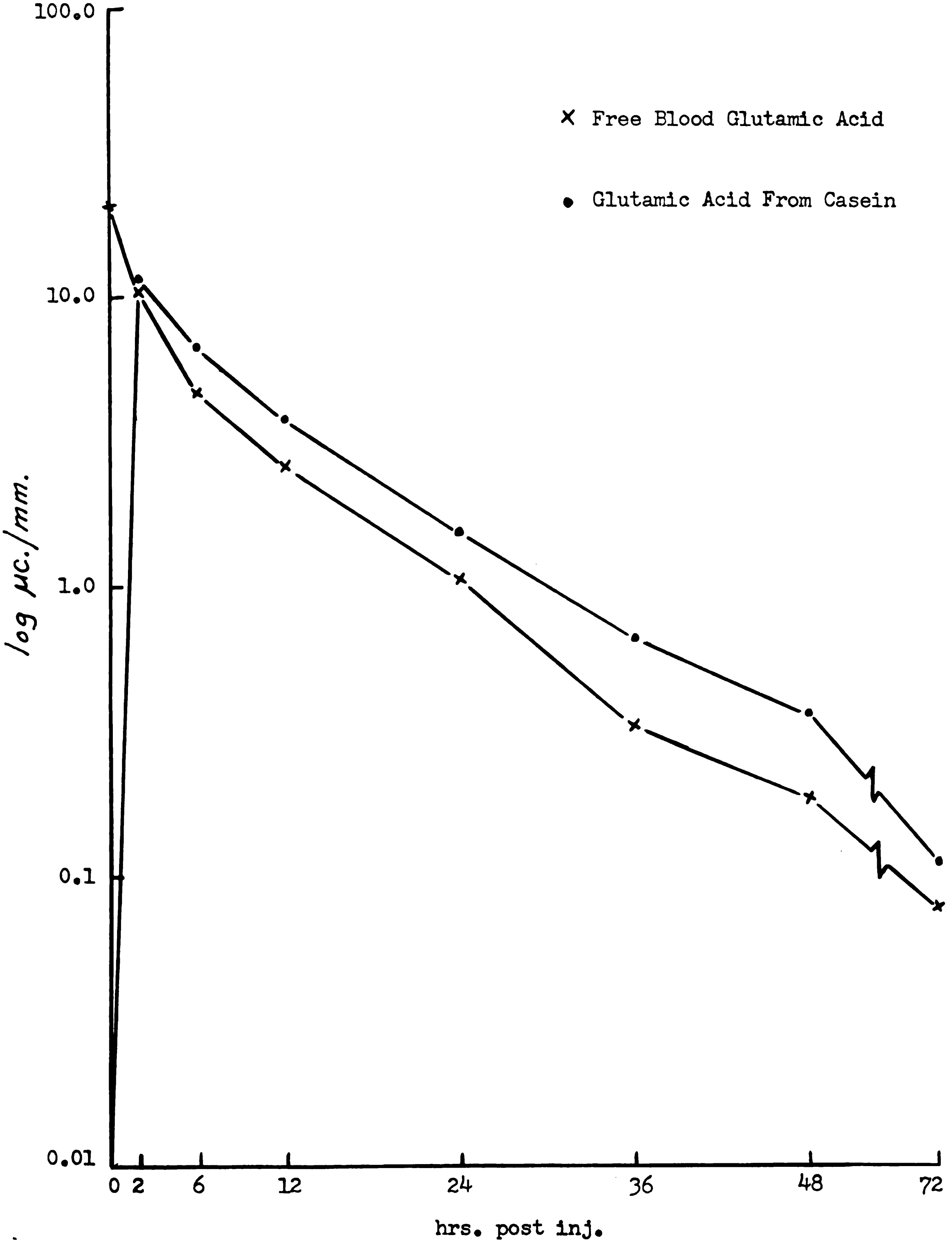

FIGURE 42. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM CASEIN ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

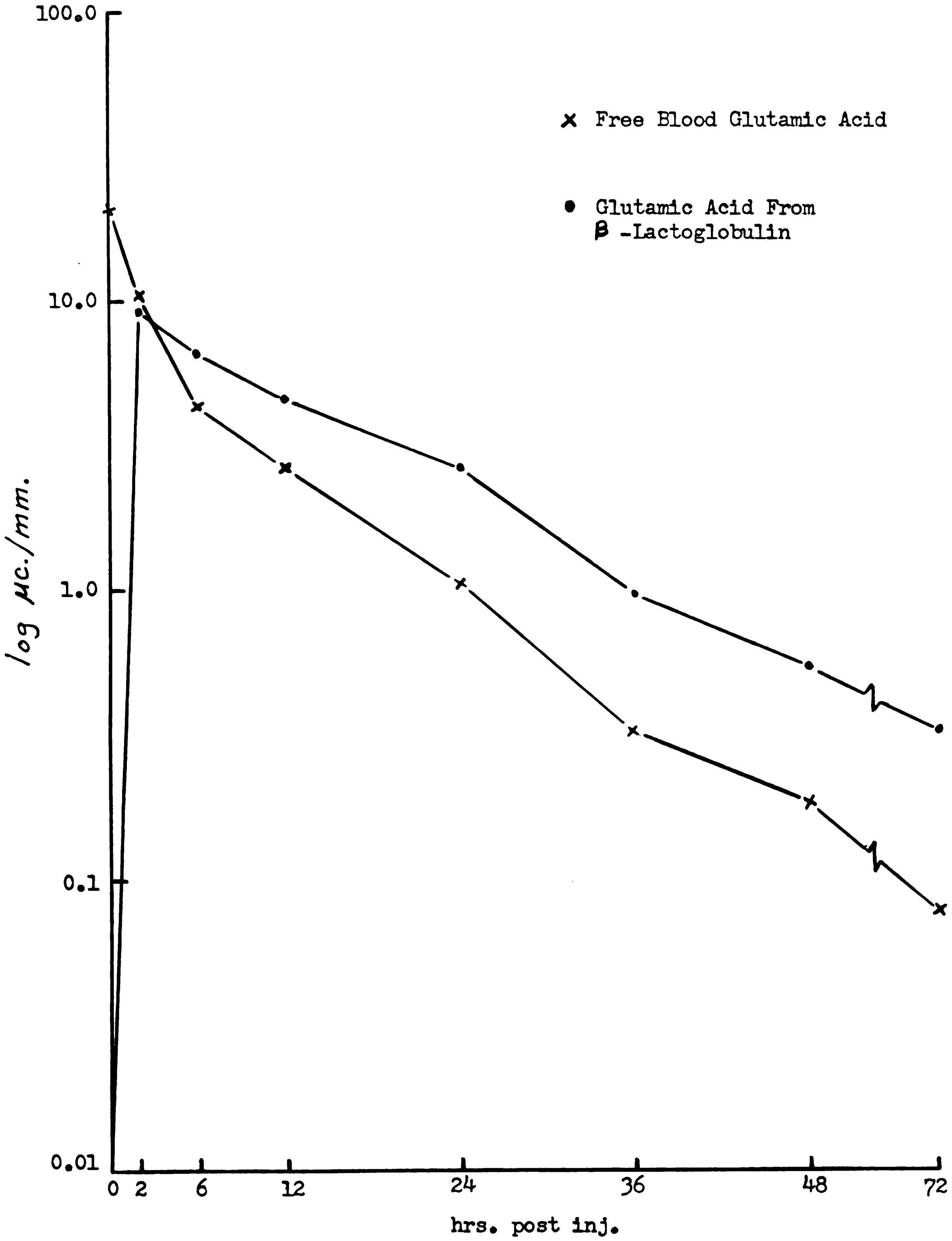

FIGURE 43. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM β-LACTOGLOBULIN ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 µC. OF DL-GLUTAMIC ACID-2-C^{14}).

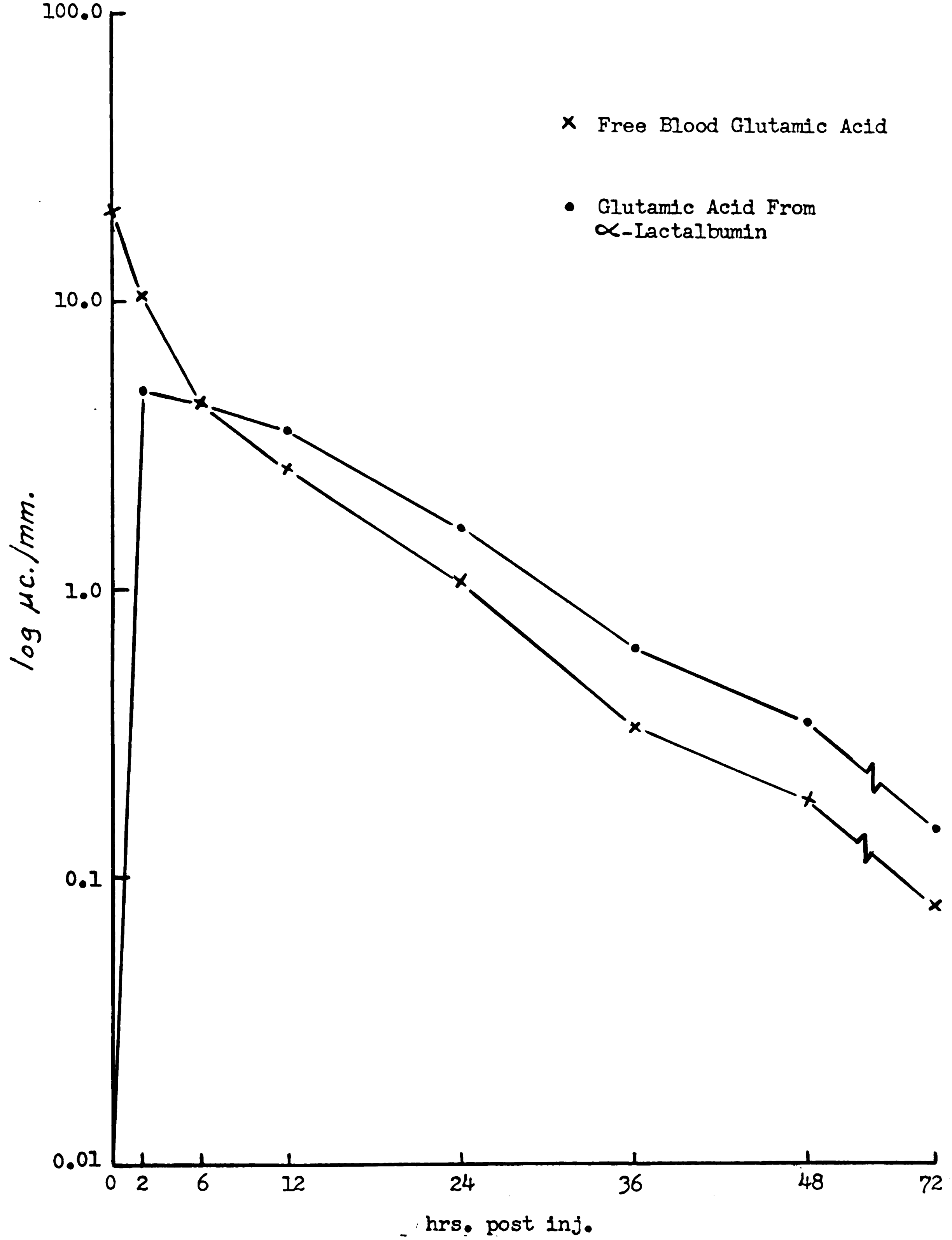

FIGURE 44. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM ∝-LACTALBUMIN ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

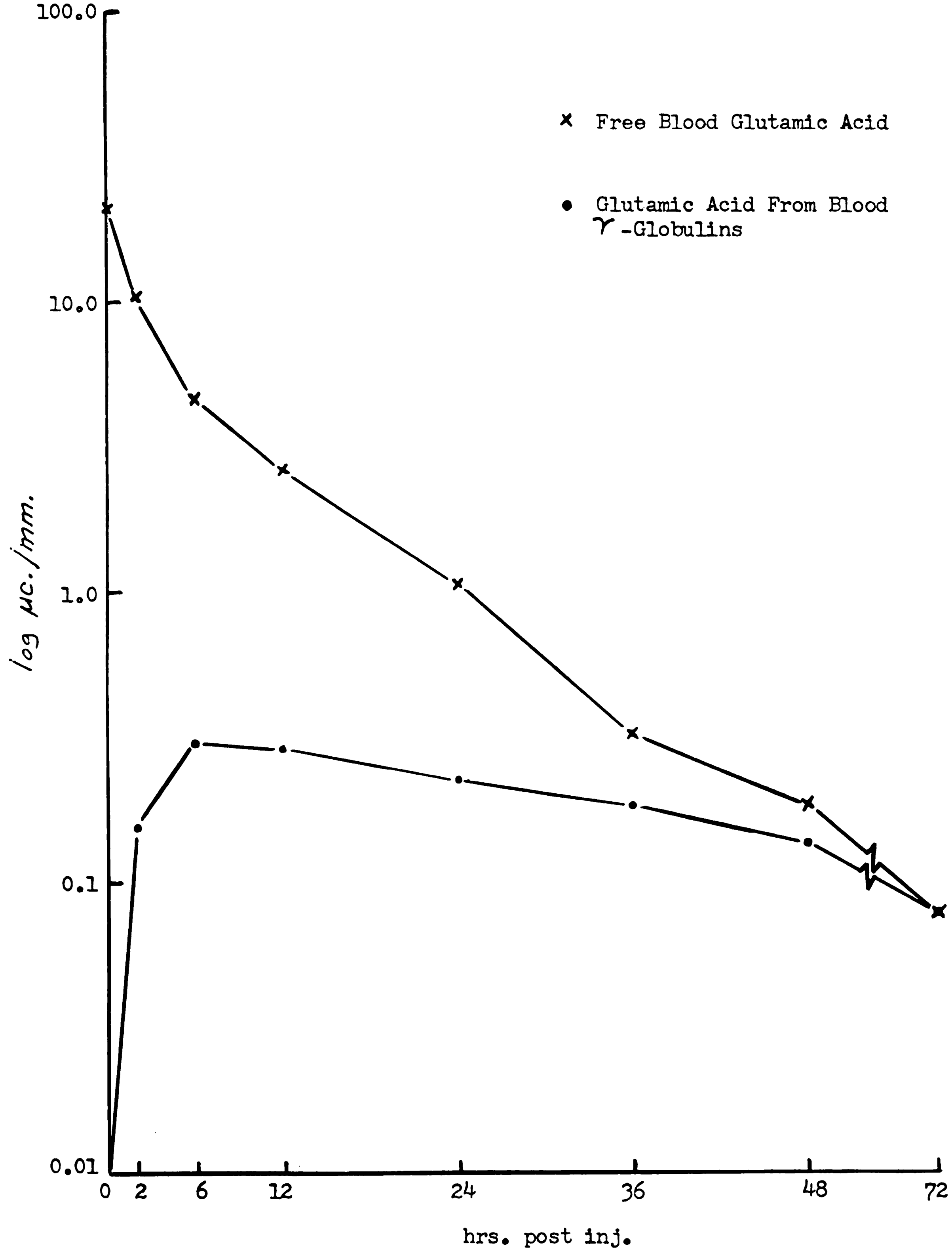

FIGURE 45. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM BLOOD γ-GLOBULINS ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

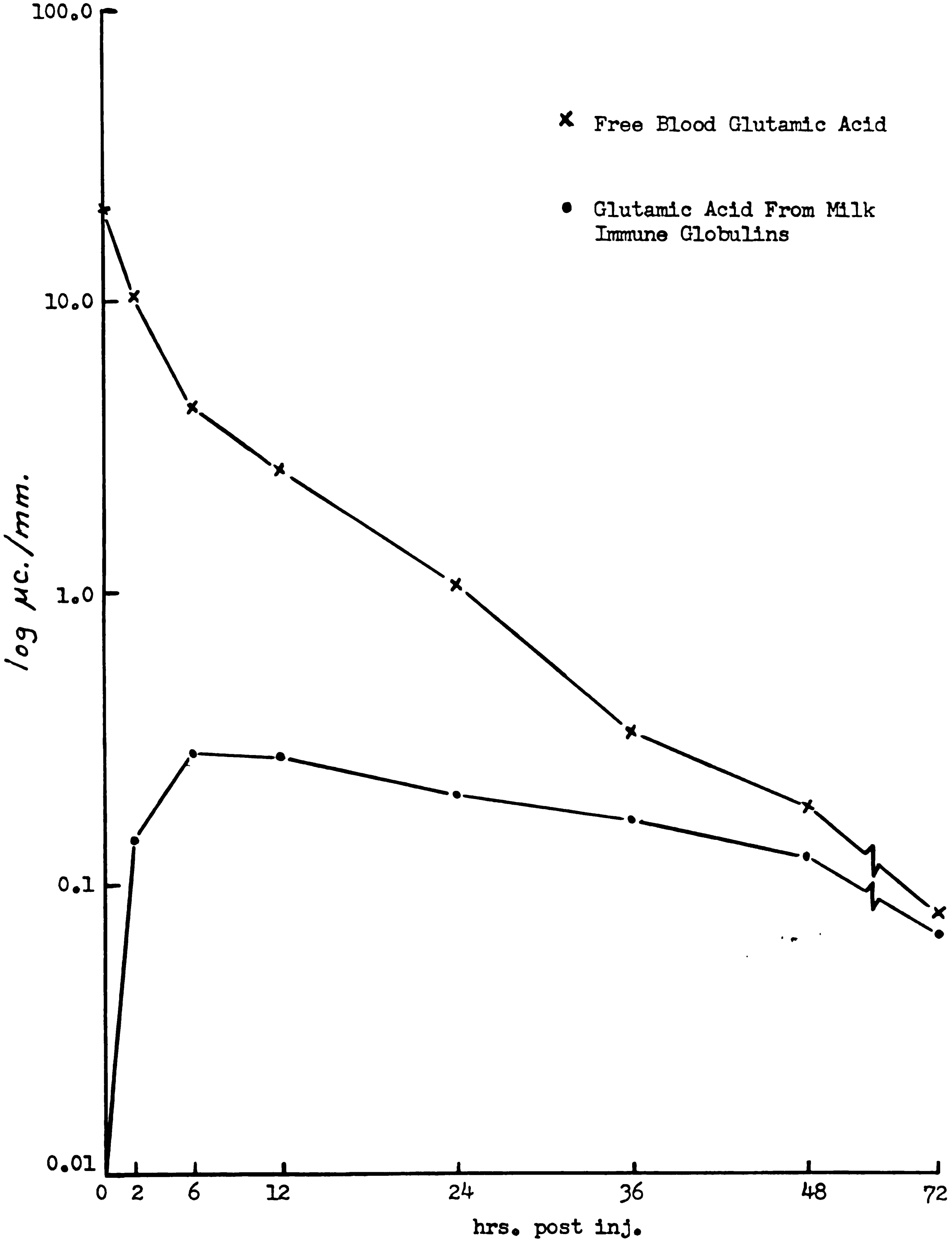

FIGURE 46. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM MILK IMMUNE GLOBULINS ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

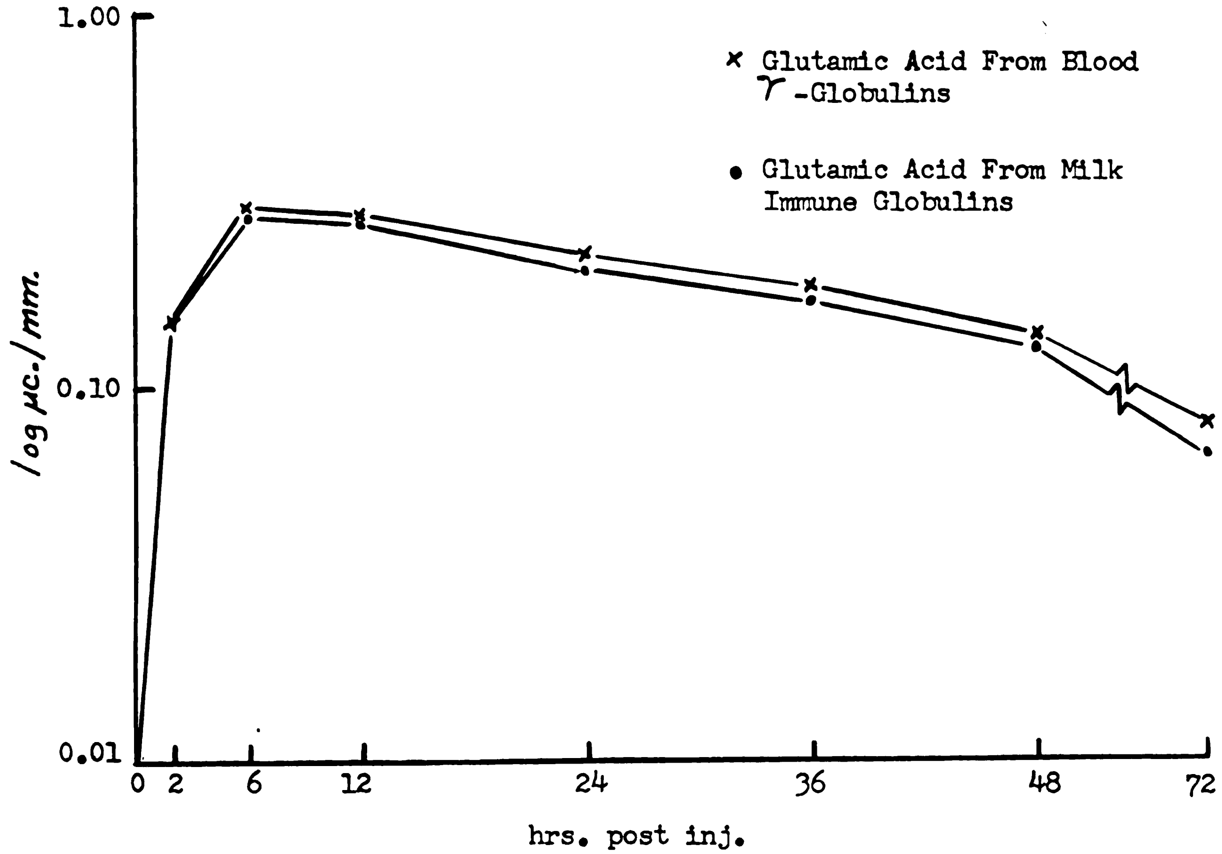

FIGURE 47. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR GLUTAMIC ACID FROM BLOOD γ-GLOBULINS AND MILK IMMUNE GLOBULINS ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

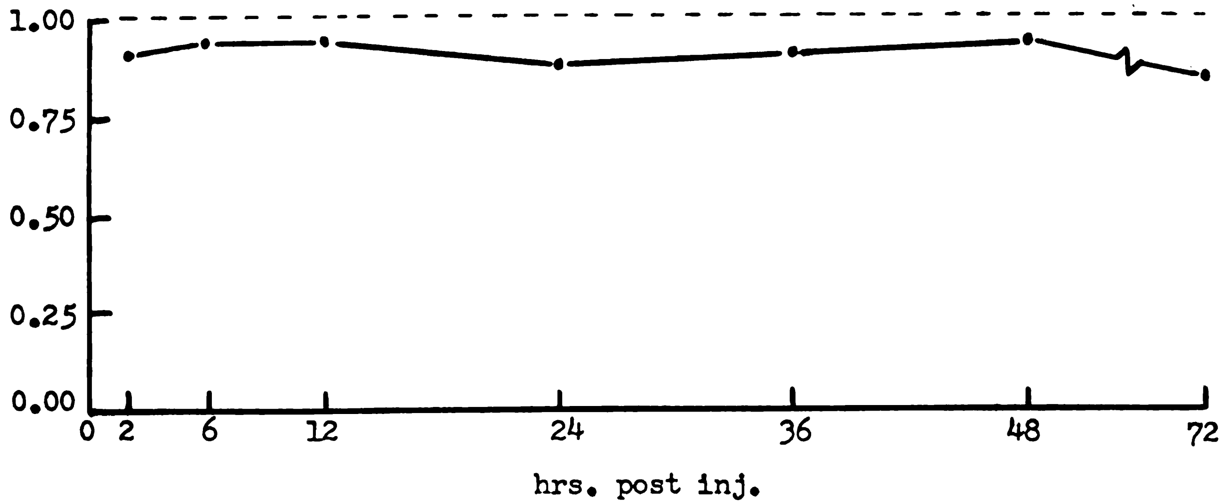

FIGURE 48. RATIOS OF THE SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM MILK IMMUNE GLOBULINS TO THE SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD γ-GLOBULINS ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

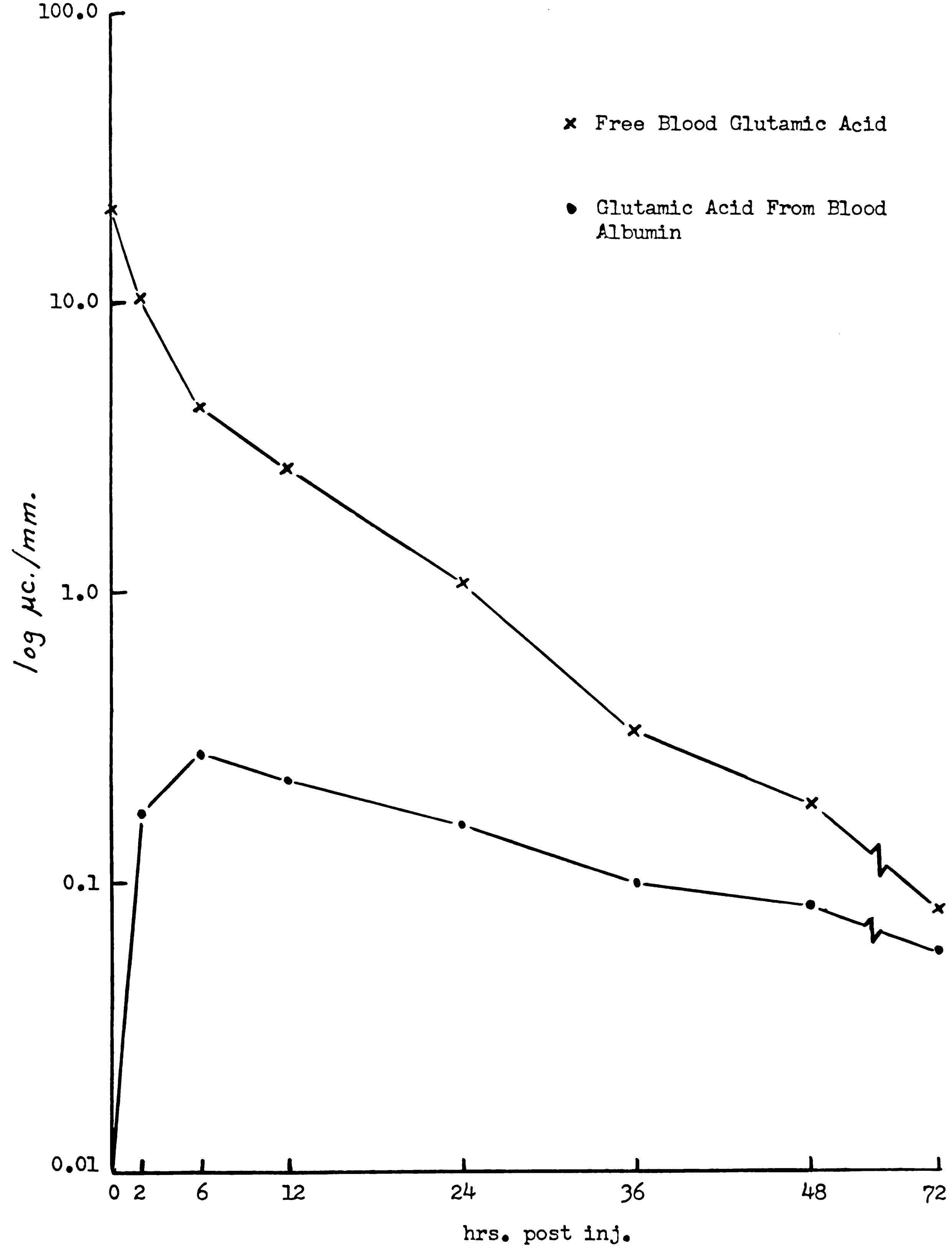

FIGURE 49. THE LOG OF THE SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM BLOOD ALBUMIN ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

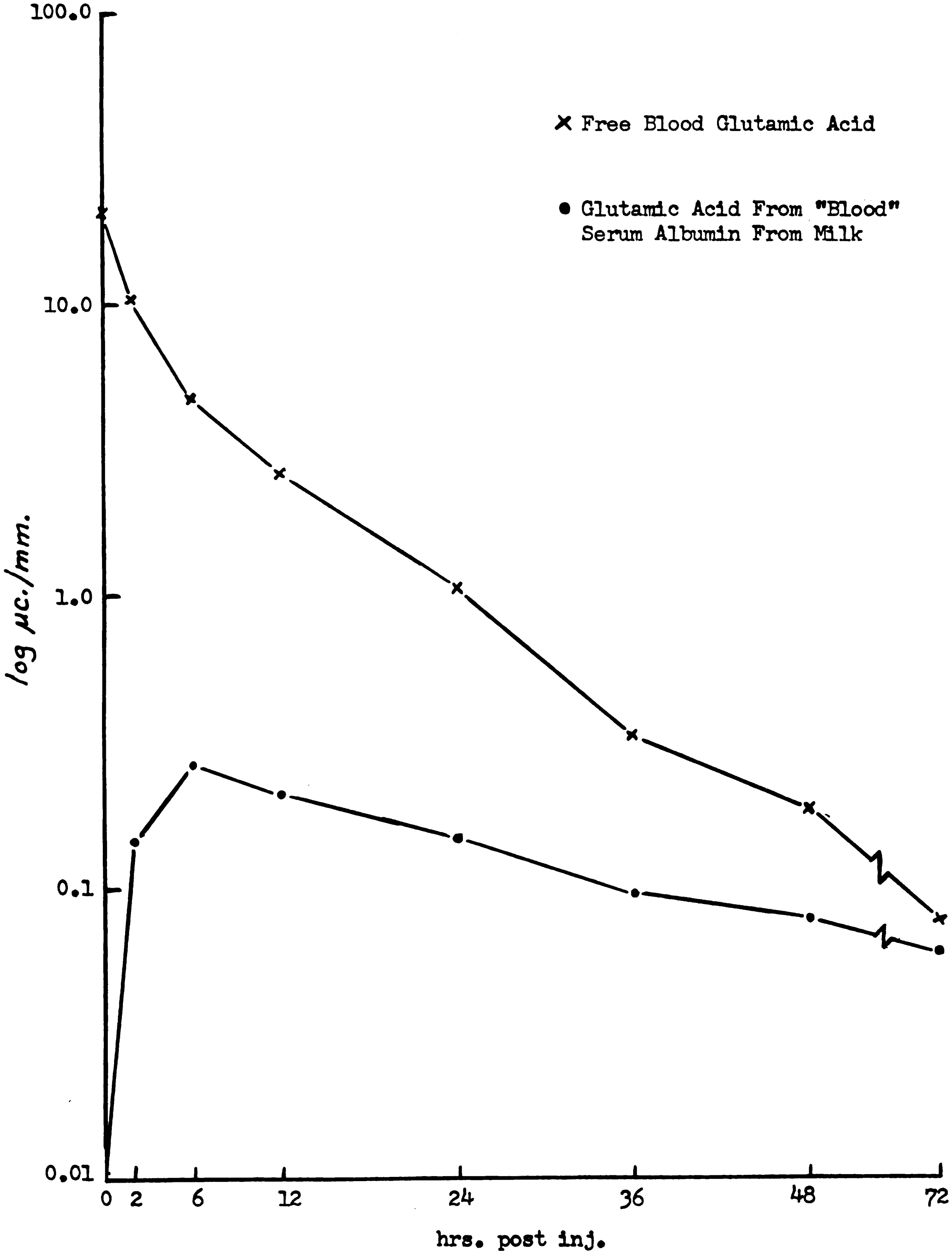

FIGURE 50. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

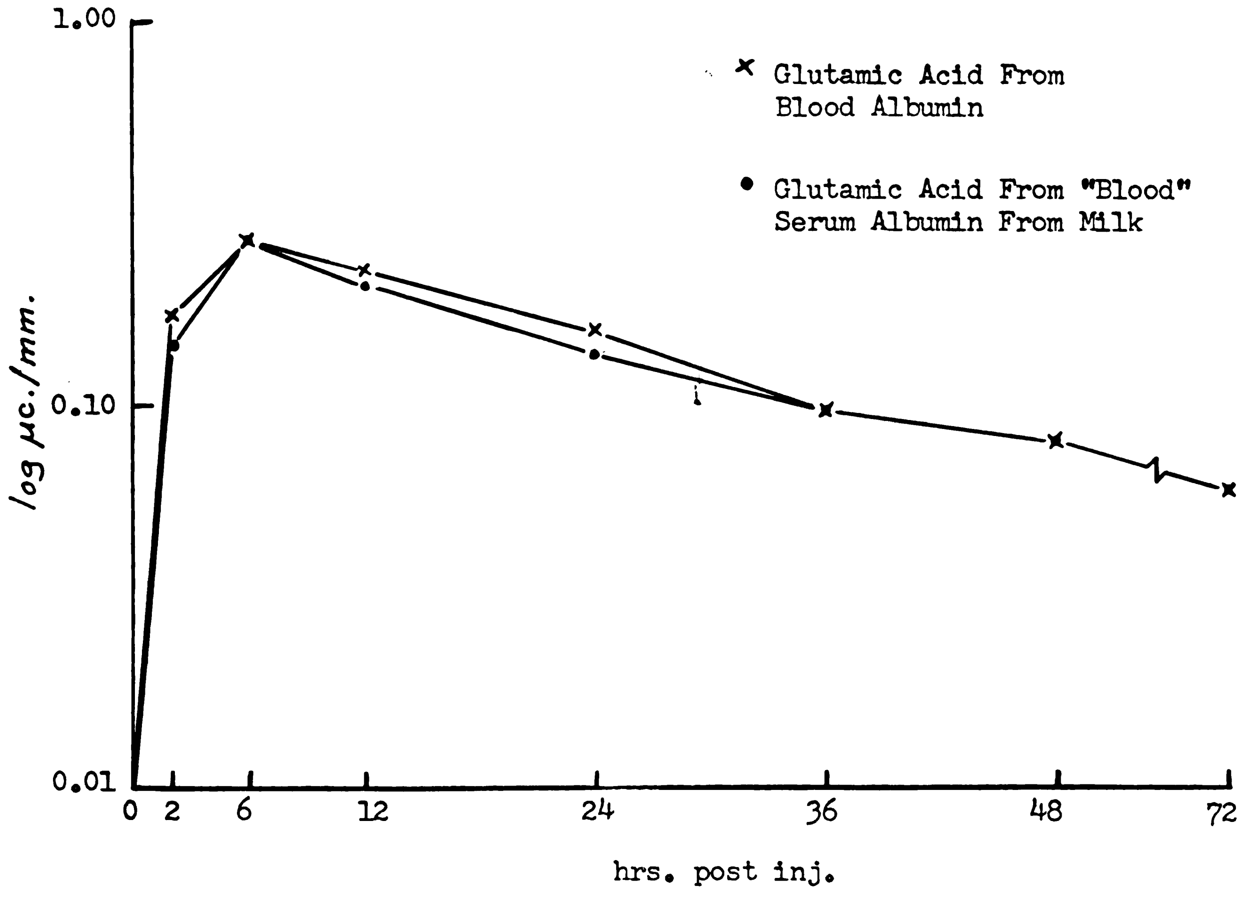

FIGURE 51. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR GLUTAMIC ACID FROM BLOOD ALBUMIN AND "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

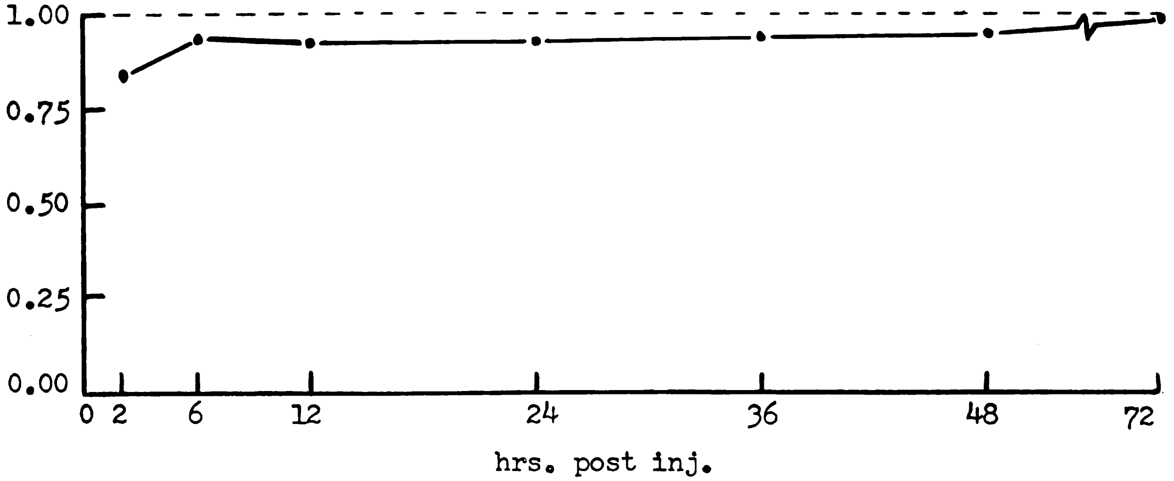

FIGURE 52. RATIOS OF THE SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM "BLOOD" SERUM ALBUMIN FROM MILK TO THE SPECIFIC ACTIVITIES OF GLUTAMIC ACID FROM BLOOD ALBUMIN ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

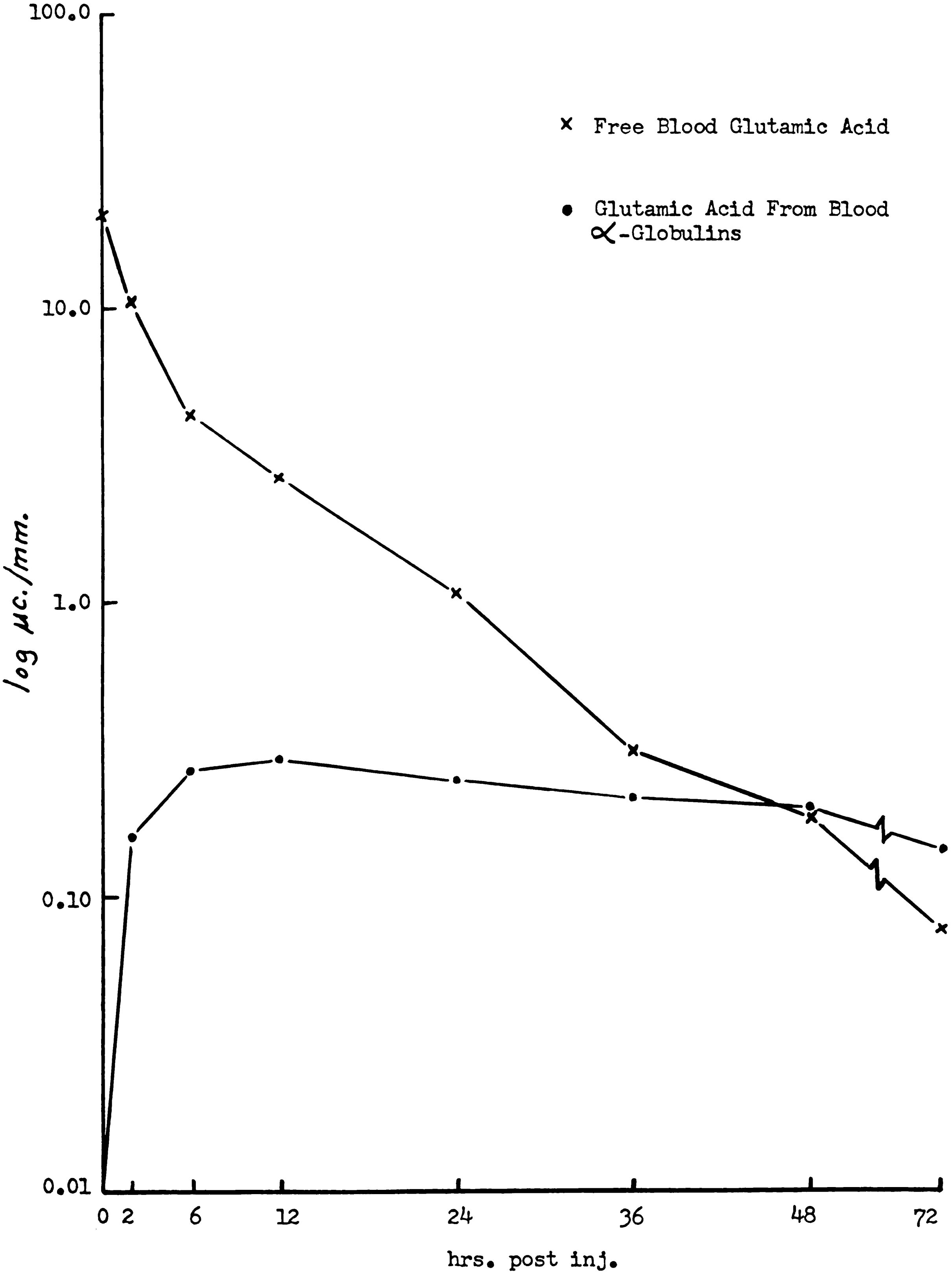

FIGURE 53. THE LOG OF THE SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM BLOOD α-GLOBULINS ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 µC. OF DL-GLUTAMIC ACID-2-C^{14}).

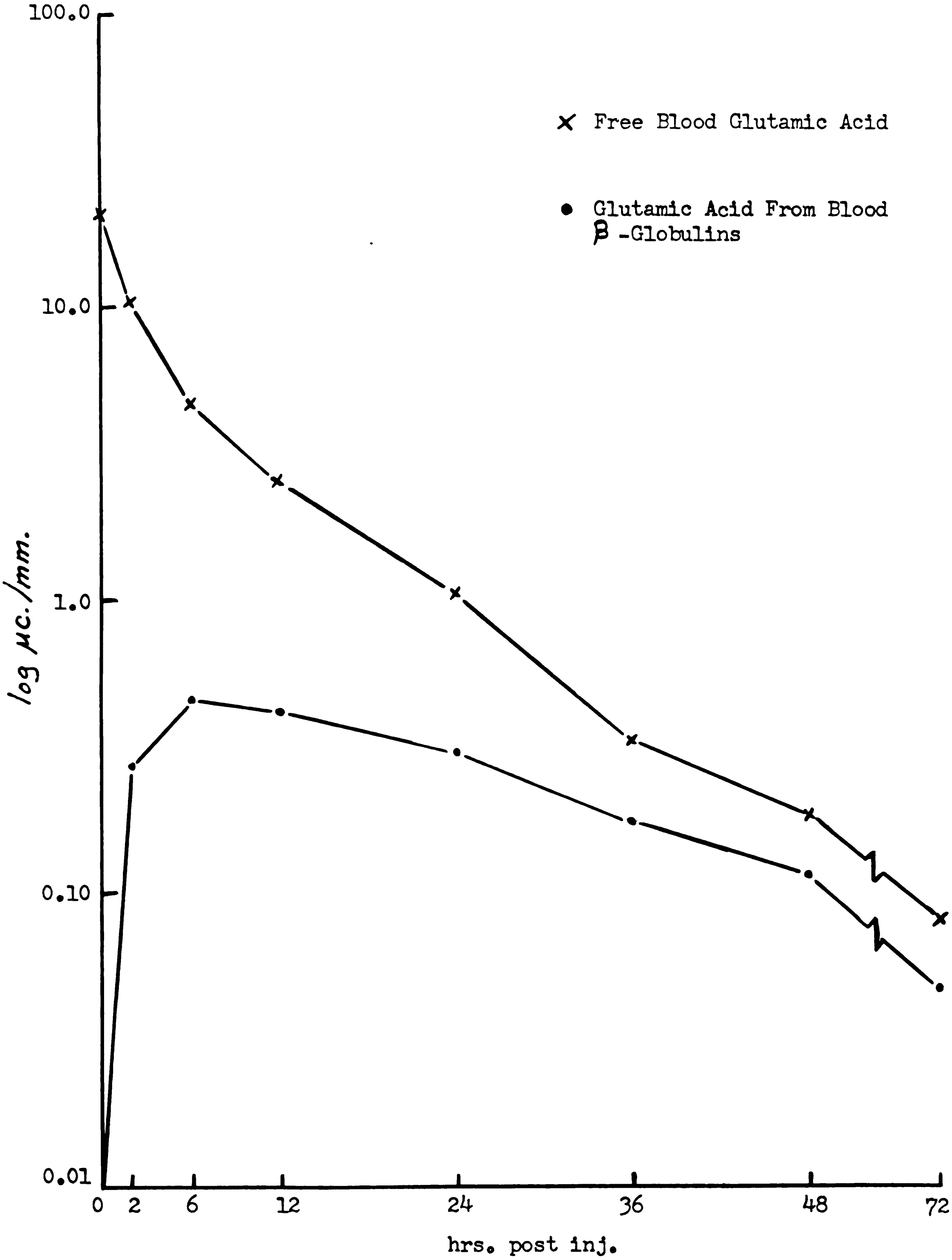

FIGURE 54. THE LOG OF SPECIFIC ACTIVITY (uc./mm.) VS. TIME (hrs. post inj.) FOR FREE BLOOD GLUTAMIC ACID AND GLUTAMIC ACID FROM BLOOD β-GLOBULINS ISOLATED FROM X-34 (LACTATING RABBIT 14TH-17TH DAYS POSTPARTUM INJECTED WITH 150.0 μC. OF DL-GLUTAMIC ACID-2-C^{14}).

Preliminary work indicated that an injection of 50 µc. of glutamic acid-2-C^{14} was too low to give sufficient labeling in the milk proteins of lactating rabbits. The levels of activity were so low that the data could not be evaluated. This is the reason that such high levels of activity were injected into these animals. The extremely rapid decline in the specific activity of free blood glutamic acid can be attributed to an extremely rapid metabolism of glutamic acid in the rabbit. This point should also be considered along with the other points discussed earlier for the rapid decline in specific activity of free blood leucine.

There are no other data available on the incorporation of glutamic acid into milk proteins by the rabbit. These data indicate that the lactating rabbit incorporates a greater percentage of free blood glutamic acid into caseins and β-lactcglobulin than the goat (Barry, 1958). There are no other data available indicating that the glutamic acid of α-lactalbumin comes directly from free glutamic acid in the blood of the rabbit.

C. Experiments 5 and 6. Experiments Designed to Demonstrate the Transfer of Blood γ-Globulins to Mammary Secretions of the Rabbit

When a free amino acid is injected directly into the blood stream, the possibility exists that it could have been incorporated into the milk immune globulins within the cells of the mammary tissue itself. This possibility has been suggested by Azimov (1959), McCarthy _et al._ (1949), and others who have injected possible precursors directly into the glands of lactating animals via the teat.

Tables 13 and 14 present the specific activity in µc./mg. of protein isolated from two lactating rabbits injected with C^{14} labeled γ-globulins. These data substantiate the previous work reported on the injection of labeled leucine and glutamic acid. However, there are some differences. In particular, it appears that there are some differences in the time required to establish equilibrium between the γ-globulins in the blood and the immune globulins of the milk (Figures 55 and 56). This might be explained by the time required for the γ-globulins within the vascular system to equilibrate with those of the interstitial fluids and the time required to pass through the epithelium of the mammary gland and enter the mammary secretions.

It is also apparent from the data presented in Tables 15 and 16 and from Figures 57 and 58 that there is a difference in the equilibration time between these two animals. One appeared to be in equilibrium shortly after 2 hours, while the other required somewhere between 6 and 12 hours. This is difficult to explain. It could possibly be explained on the basis of individual variation between these two particular animals, one being extremely rapid while the other

TABLE 13. THE SPECIFIC ACTIVITIES OF BLOOD γ-GLOBULINS AND MILK IMMUNE GLOBULINS FROM A LACTATING RABBIT (NO. X-18, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 0.31 μC. (400 mg.) C^{14} LABELED γ-GLOBULINS.

hrs. post inj.	DPM	mg. protein counted	μc./mg. protein x 10^4
		Blood γ-Globulins	
0.16	13,410	35.6	1.70
2	13,725	38.9	1.59
6	9,635	30.5	1.44
12	10,945	39.4	1.25
24	9,950	44.9	1.00
48	9,145	47.8	0.86
72	7,700	50.0	0.78
		Milk Immune Globulins	
2	14,100	44.8	1.42
6	11,615	38.5	1.36
12	9,475	35.1	1.22
24	6,540	41.6	0.90
48	8,948	48.9	0.83
72	8,130	49.5	0.74

TABLE 14. THE SPECIFIC ACTIVITIES OF BLOOD γ-GLOBULINS AND MILK IMMUNE GLOBULINS FROM A LACTATING RABBIT (NO. X-19, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 2.0 μC. (800 mg.) C^{14} LABELED γ-GLOBULINS.

hrs. post inj.	DPM	mg. protein counted	μc./mg. protein x 10^4
		Blood γ-Globulins	
0.16	61,950	39.3	7.10
2	50,650	38.4	5.97
6	41,975	37.3	5.07
12	28,790	28.9	4.48
24	27,625	32.2	3.91
48	24,595	34.6	3.20
72	22,850	36.5	2.90
		Milk Immune Globulins	
2	23,800	38.4	2.79
6	29,410	37.3	3.55
12	26,945	28.9	4.10
24	20,925	26.1	3.61
48	28,600	40.0	3.22
72	21,850	35.2	2.81

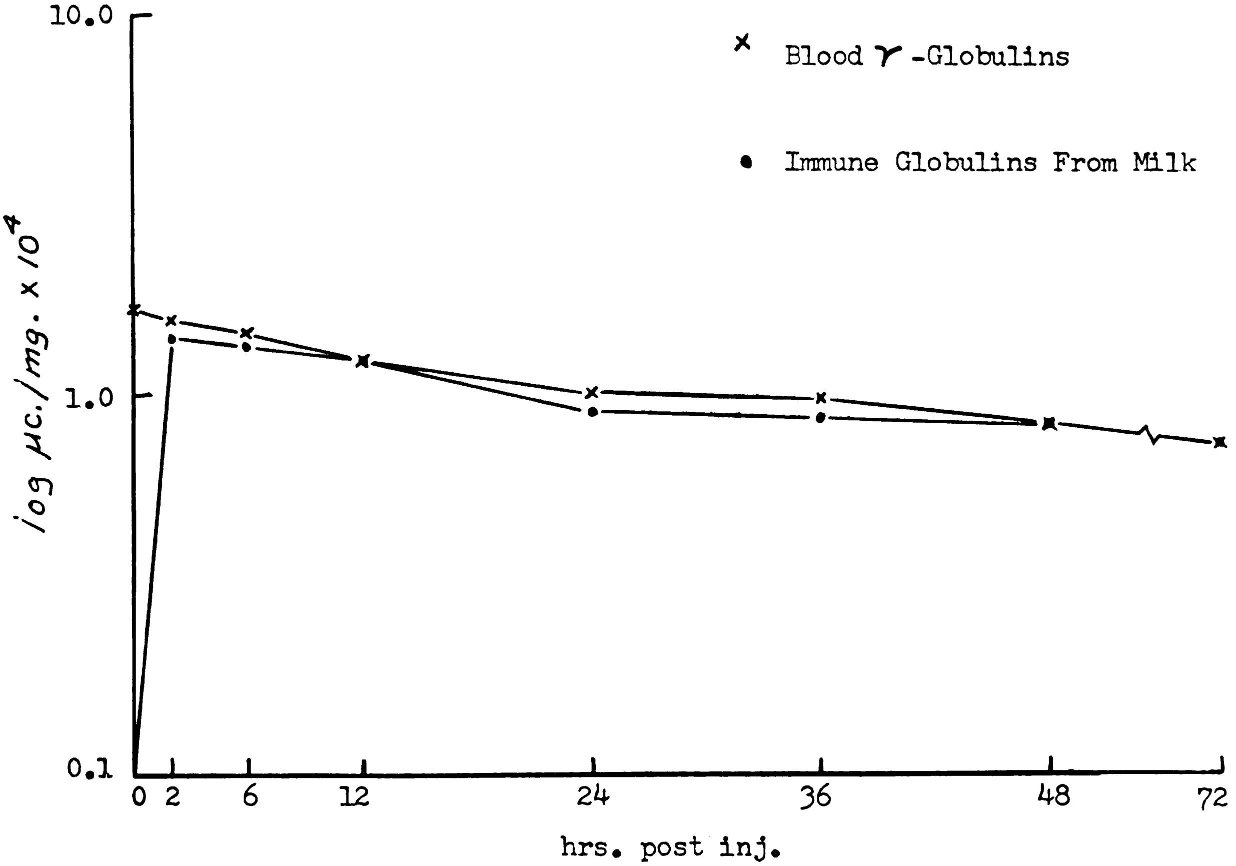

FIGURE 55. THE LOG OF THE SPECIFIC ACTIVITY (uc./mg. x 10^4) VS. TIME (hrs. post inj.) FOR BLOOD γ-GLOBULINS AND IMMUNE GLOBULINS FROM MILK ISOLATED FROM X-18 LACTATING RABBIT 10TH-13TH DAYS POSTPARTUM INJECTED WITH 0.13 μC. C^{14} LABELED γ-GLOBULIN).

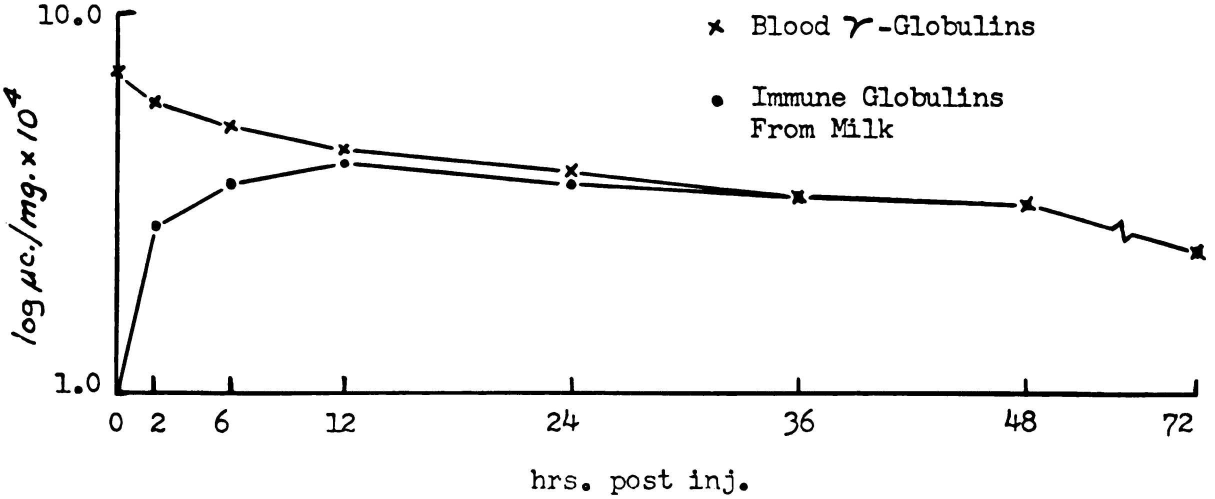

FIGURE 56. THE LOG OF THE SPECIFIC ACTIVITY (uc./mg. x 10^4) VS. TIME (hrs. post inj.) FOR BLOOD γ-GLOBULIN AND IMMUNE GLOBULINS FROM MILK ISOLATED FROM X-19 (LACTATING RABBIT 10TH-13TH DAYS POSTPARTUM INJECTED WITH 2.0 μC. OF C^{14} LABELED γ-GLOBULINS).

TABLE 15. RATIOS OF THE SPECIFIC ACTIVITIES OF THE (a) MILK IMMUNE GLOBULINS TO THE (b) SPECIFIC ACTIVITIES OF BLOOD γ-GLOBULINS IN A LACTATING RABBIT (NO. X-18, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 0.31 μC. (400 mg.) C^{14} LABELED γ-GLOBULINS.

hrs. post inj.	a/b	% of milk immune globulins derived from blood γ-globulins
2	0.894	---
6	0.945	94.5
12	0.975	97.5
24	0.900	90.0
48	0.965	96.5
72	0.950	95.0
	0.945 ± 0.0129[1]	

[1] The mean value of the ratios from 6 through 72 hours is presented with standard error.

TABLE 16. RATIOS OF THE SPECIFIC ACTIVITIES OF THE (a) MILK IMMUNE GLOBULINS TO THE (b) SPECIFIC ACTIVITIES OF BLOOD γ-GLOBULINS IN A LACTATING RABBIT (NO. X-19, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 2.0 μc. (800 mg.) C^{14} LABELED γ-GLOBULINS.

hrs. post inj.	a/b	% of milk immune globulins derived from blood γ-globulins
2	0.467	---
6	0.700	---
12	0.916	91.6
24	0.925	92.5
48	1.005	100.0
72	0.970	97.0
	0.954 ± 0.0161[1]	

[1] The mean value of the ratios from 12 through 72 hours is presented with standard error.

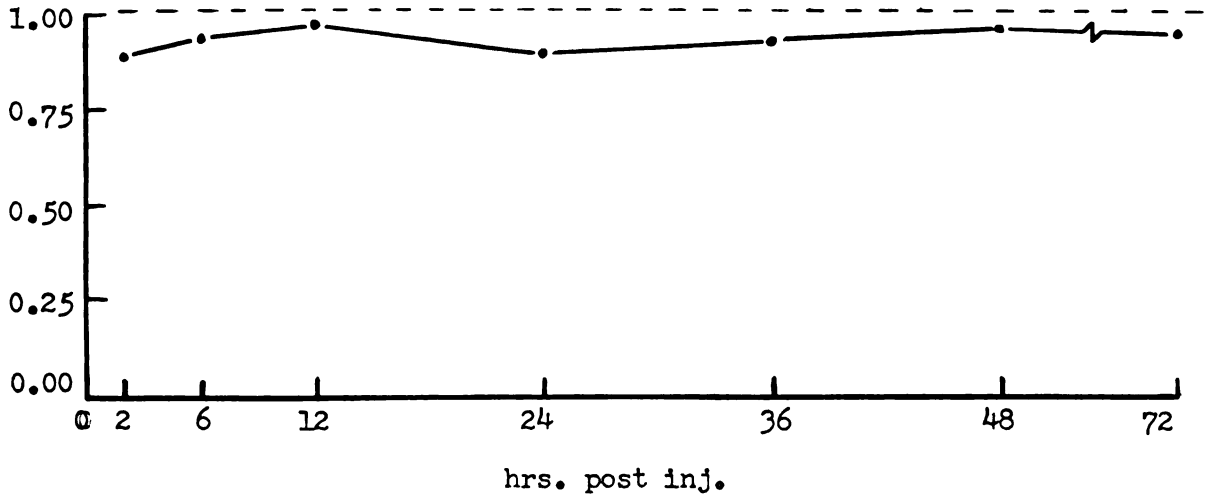

FIGURE 57. RATIOS OF THE SPECIFIC ACTIVITIES OF IMMUNE GLOBULINS FROM MILK TO THE SPECIFIC ACTIVITIES OF γ-GLOBULINS IN BLOOD ISOLATED FROM X-18 (LACTATING RABBIT 10TH-13TH DAYS POST-PARTUM INJECTED WITH 0.31 μC. OF C^{14} LABELED γ-GLOBULINS).

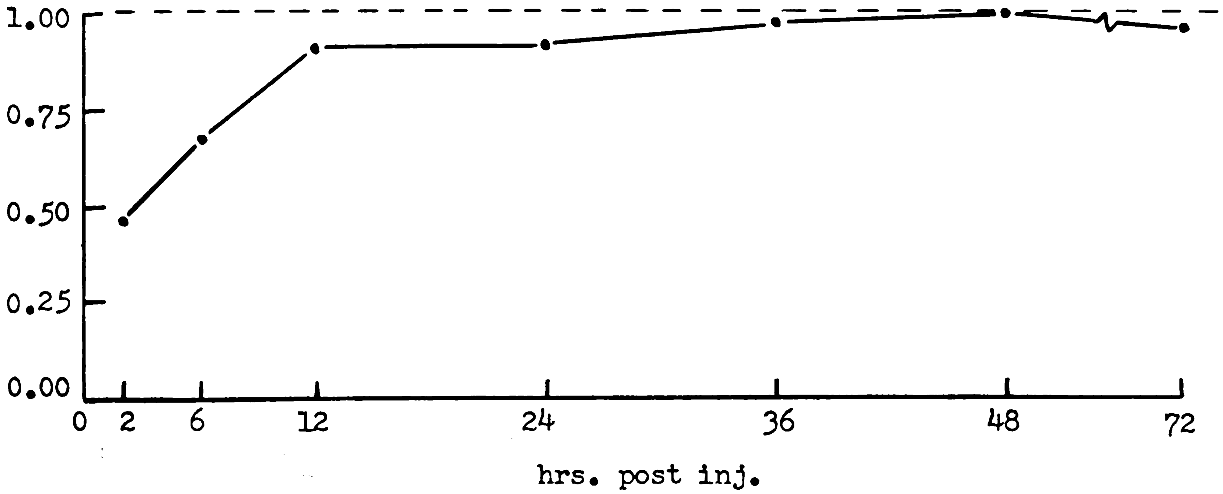

FIGURE 58. RATIOS OF THE SPECIFIC ACTIVITIES OF IMMUNE GLOBULINS FROM MILK TO THE SPECIFIC ACTIVITIES OF γ-GLOBULINS IN BLOOD ISOLATED FROM X-19 (LACTATING RABBIT 10TH-13TH DAYS POST-PARTUM INJECTED WITH 2.0 μC. OF C^{14} LABELED γ-GLOBULINS).

one is very slow. It is possible that the slow one was affected by some disease or malfunction. However, all animals were carefully checked and judged to be healthy before being utilized for experimentation. Regardless of this deviation in the equilibration time, it is obvious that after equilibration was reached, there was a very close relationship between the specific activity of the blood γ-globulin and the immune globulin of the milk.

The means of the ratios are presented after equilibration had been reached. Tables 15 and 16 indicate that after equilibration, 93.4% of the immune globulins in the milk of one animal and 93.8% of the immune globulins in the milk of the other animal were derived from the blood γ-globulin. Due to the very close agreement of the specific activities of these protein fractions, it can be assumed that in the case of these two animals the immune globulins were not synthesized within the mammary tissues but came directly from the blood γ-globulins. This does not preclude the possibility that immune globulins or γ-globulins, as the case may be, can be synthesized within the mammary gland itself. Petersen _et al._ (1958) has stated that cows injected with antigens of various kinds directly into the mammary gland via the teat will produce specific antibodies within the milk. Also, Askonas and Humphrey (1958) have shown local production of antibodies when a tissue is injected with an antigen. However, these data and the results with labeled leucine and glutamic acid provide good evidence that the normal, healthy mammary gland does not produce the immune globulins of milk. It just transfers them from the interstitial fluid pool to the milk.

Smith (1948) reported that the percent of leucine in the immune globulins of cow's milk is about 20% higher than in the γ-globulins of the blood. This would mean an incorporation of 2 additional mg. of leucine per 100 mg. of milk immune globulins by the mammary tissue. This would cause a dilution of the specific activity (μc./mg. of protein) of the milk immune globulins and thus yield values different from the specific activities of blood γ-globulins. The work of Larson and Gillespie (1957) and Larson (1958) has shown that β_2-globulins are also transferred to the immune globulins of cow's milk. The findings of Smith (1948), Larson and Gillespie (1957) and Larson (1958) do not appear to be true in the case of these two rabbits. It seems that the specific activities of the immune globulins of the milk of these two animals would have been different from the specific activities of the γ-globulins of the blood if the non-labeled β_2-globulins and additional non-labeled leucine had been diluting the immune globulins in the rabbits' milk. There appears to be a species difference between the rabbit and cow.

Fink _et al._ (1944) were the first to inject labeled proteins into the living animal to study their behavior _in vivo_. Since that time, many studies have been done using I^{131}, C^{14} and N^{15} labeled plasma proteins and purified proteins to study their distribution in body fluids, turnover rates, and biological half-times. Dixon _et al._ (1952), Dovey _et al._ (1954), Germuth _et al._ (1951), and Cohen _et al._ (1956) have reported half-times for γ-globulins injected into rabbits ranging from 3.2 to 5.7 days. Inspection of the graphs for the specific activity of blood γ-globulins in Figures 55 and 56 reveals a half-time of 2 to 3 days as determined by the graphic method. This is not unusual, since the γ-globulins were leaving the body via the mammary secretions in this case. The data of the investigators above were reported on non-lactating rabbits. The slope of these curves is greater during the first few hours post injection. This is due to equilibration of the C^{14}-γ-globulin with the tissue fluids. Wasserman and Meyerson (1951) reported that equilibration of injected I^{131} labeled albumin required 7 hours for complete equilibration into the lymph and interstitial fluid in the dog. It appears that the equilibration of γ-globulin in the rabbit was a little faster.

The work presented in Tables 13 and 14 is the only example of homologous C^{14}-γ-globulin injected intravenously into lactating rabbits and the subsequent isolation of immune globulins of milk with the same specific activity. On the basis of this study and the results presented earlier, it has been shown that the immune globulins of rabbit milk are derived directly from the γ-globulins of the blood. These conclusions are in agreement with conclusions reached from studies on the cow.

Additional data on the specific activities of various blood and milk protein fractions after injection of C^{14}-γ-globulin are presented in Tables 6 and 7 in the appendix. The activity in these fractions is very low and of little significance. It appears to be due to amino acids released from the catabolism of the injected radioactive γ-globulin.

D. Experiments 7 and 8. Experiments Designed to Demonstrate the Transfer of Blood Albumins to Mammary Secretions of the Rabbit

Since the experiments with C^{14}-γ-globulin demonstrated clearly the transfer of this protein to mammary secretions, the experiment was repeated using C^{14}-albumins. Tables 17 and 18 and Figures 59 and 60 present the specific activities of blood albumins and "blood" serum albumins from milk of the two lactating rabbits used in these experiments. These data show that after equilibration with the interstitial fluids, there is remarkable agreement between the specific activities of the blood albumin and the "blood" serum albumin in milk. In these experiments, the equilibration time appears to be somewhere between 6 and 12 hours for both animals. This is a little longer than in the case of the injected γ-globulins. This is interesting in view of the fact that the molecular weight of albumin is 60,000 to 70,000 and the molecular weight of the blood γ-globulin is around 160,000. It should be remembered that the negative charge on blood albumin is greater than the charge on γ-globulin, since the mobility of albumin is much greater than that of γ-globulin during electrophoresis at pH 8.6. This might explain some of the difference in the time of equilibration.

It is postulated that there is some mechanism in the epithelial cells of the mammary gland which is responsible for the above difference. This is not only evident because of the time relationship but also because of the quantitative differences. The albumin fraction of blood is considerably larger than the γ-globulin fraction of blood. The reverse is true in the milk. The immune globulin fraction of the milk is much greater than the "blood" serum albumin fraction. If these two proteins were entering the mammary secretions by the same mechanism, it

TABLE 17. THE SPECIFIC ACTIVITIES OF BLOOD ALBUMIN AND "BLOOD" SERUM ALBUMIN FROM MILK FROM A LACTATING RABBIT (NO. X-17, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 5.16 µC. (2.0 gm.) C^{14} LABELED ALBUMIN.

hrs. post inj.	DPM	mg. protein counted	µc./mg. protein x 10^4
		Blood Albumin	
0.16	12,480	40.0	1.47
2	13,000	44.9	1.30
6	11,835	47.8	1.12
12	10,400	46.3	1.02
24	9,348	48.1	0.88
48	6,335	42.5	0.67
72	5,340	49.0	0.47
	"Blood" Serum Albumin From Milk Serum		
2	615.0	0.34	0.82
6	404.0	0.18	1.00
12	518.0	0.24	0.98
24	429.5	0.27	0.72
48	620.0	0.40	0.70
72	12.0[1]	0.12	0.45

---

[1] The count of this sample was not significantly different from background.

TABLE 18. THE SPECIFIC ACTIVITIES OF BLOOD ALBUMIN AND "BLOOD" SERUM ALBUMIN FROM MILK FROM A LACTATING RABBIT (NO. X-20, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 13.2 µC. (3.0 gm.) C^{14} LABELED ALBUMIN.

hrs. post inj.	DPM	mg. protein counted	µc./mg. protein x 10^4
		Blood Albumin	
0.16	22,695	33.6	3.05
2	29,250	50.2	2.65
6	24,795	48.5	2.30
12	19,915	41.6	2.15
24	19,505	45.0	1.95
48	15,600	39.3	1.79
72	14,990	47.0	1.35
		"Blood" Serum Albumin From Milk Serum	
2	140.0	0.40	1.58
6	83.5	0.21	1.79
12	126.0	0.27	2.11
24	63.0	0.15	1.94
48	148.1	0.38	1.76
72	35.2	0.12	1.32

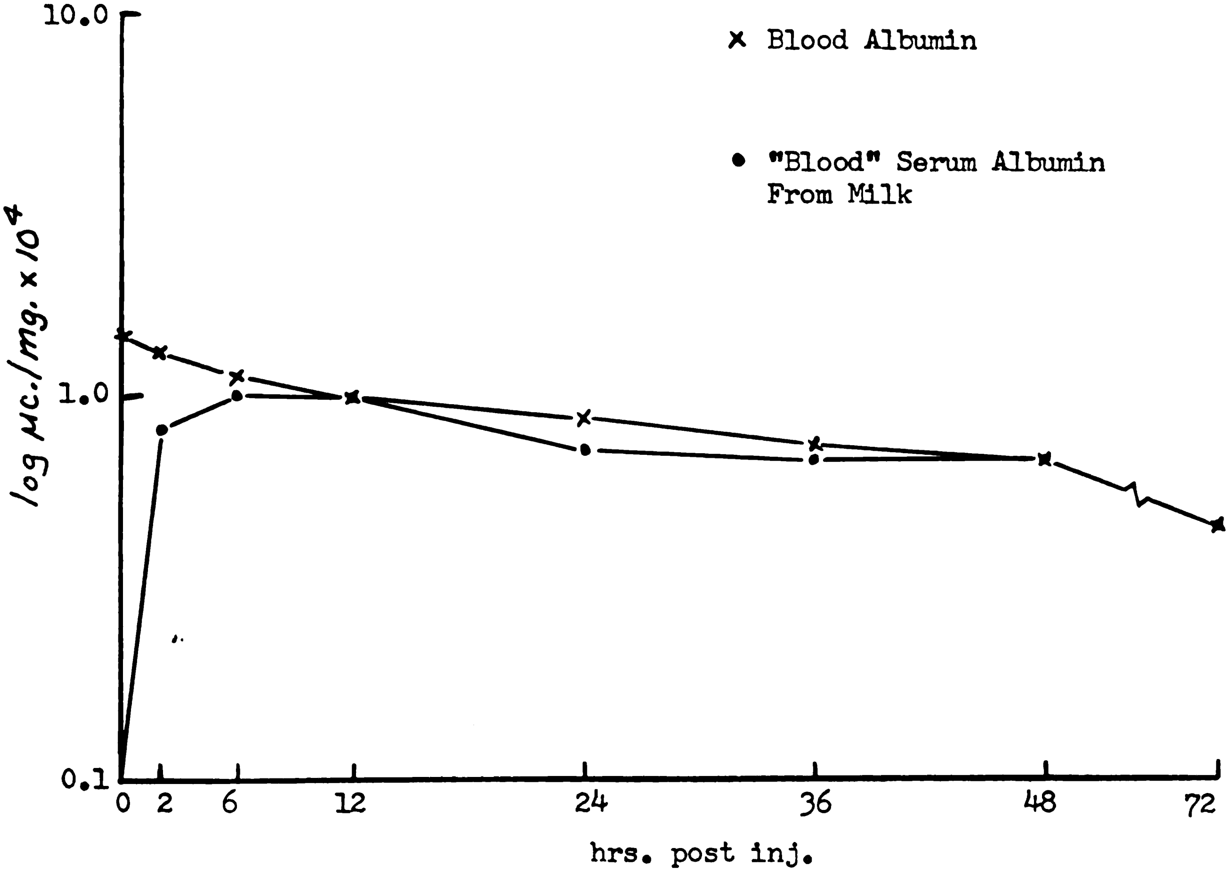

FIGURE 59. THE LOG OF THE SPECIFIC ACTIVITY (uc./mg. x 10^4) VS. TIME (hrs. post inj.) FOR BLOOD ALBUMIN AND "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-17 (LACTATING RABBIT, 10TH-13TH DAYS POSTPARTUM INJECTED WITH 5.16 μC. OF C^{14} LABELED ALBUMIN).

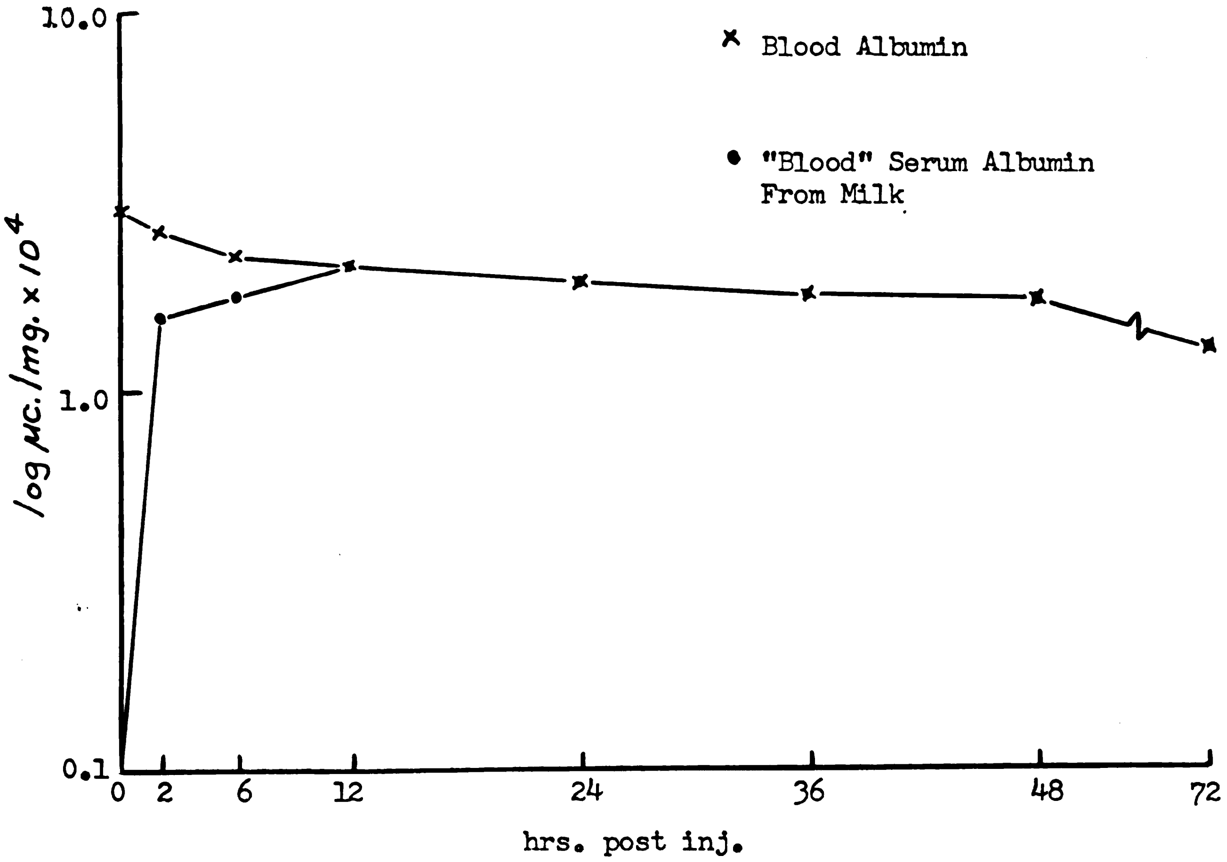

FIGURE 60. THE LOG OF THE SPECIFIC ACTIVITY (uc./mg. x 10^4) VS. TIME (hrs. post inj.) FOR BLOOD ALBUMIN AND "BLOOD" SERUM ALBUMIN FROM MILK ISOLATED FROM X-20 (LACTATING RABBIT 10TH-13TH DAYS POSTPARTUM INJECTED WITH 13.2 μC. OF C^{14} LABELED ALBUMIN).

seems highly probable that the ratio of albumin to globulin would be the same in both blood and milk. This definitely is not the case. Therefore, it is believed that the mammary tissue actively takes up the blood γ-globulins or that they pass from the blood to the mammary secretion with relative ease. Due to the extremely low concentration of "blood" serum albumin in milk, it appears that the blood albumin enters the mammary secretion in a passive manner. This could possibly be due to a hydrostatic pressure phenomena. Another possibility is that the albumin is passed from the interstitial fluid through the mammary epithelium with difficulty. This might be due to the charge on the albumin molecule. These conclusions on the incorporation of blood albumin in mammary secretions are supported by the recent work of Lecce and Legates (1959). They have reported that the levels of blood serum albumin and immune globulins increase in the cow's milk during mastitis. This may indicate a greater permeability to albumin by the mammary epithelium.

Tables 19 and 20 and Figures 61 and 62 present the ratios of the specific activities of "blood" serum albumin in milk to the specific activities of blood albumin. It is obvious that after 12 hours there is a high degree of similarity between the two protein fractions. From the means of these ratios, it can be calculated that 92.2% of the "blood" serum albumin in milk in one animal and 98.2% of the "blood" serum albumin in milk in the other animal was derived from the blood albumin after the first 12 hours post injection.

Additional data on the specific activities of other blood and milk fractions from these animals are presented in Tables 8 and 9 in the appendix. The activity in these fractions is very low and of little significance. It is due to amino acids released by the catabolism of the injected radioactive albumin.

TABLE 19. THE RATIOS OF THE (a) SPECIFIC ACTIVITIES OF "BLOOD" SERUM ALBUMIN FROM MILK TO THE (b) SPECIFIC ACTIVITIES OF BLOOD ALBUMIN FROM A LACTATING RABBIT (NO. X-17, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 5.16 µC (2.0 gm.) C^{14} LABELED ALBUMIN.

hrs. post inj.	a/b	% of "blood" serum albumin from milk derived from blood serum albumin
2	0.557	---
6	0.777	---
12	0.962	96.2
24	0.819	81.9
48	1.040	100.0
72	0.958	95.8
	0.970 ± 0.0482[1]	

---

[1] The mean value of the ratios from 12 through 72 hours post injection with standard error.

TABLE 20. THE RATIOS OF THE (a) SPECIFIC ACTIVITIES OF "BLOOD" SERUM ALBUMIN FROM MILK TO THE (b) SPECIFIC ACTIVITIES OF BLOOD ALBUMIN FROM A LACTATING RABBIT (NO. X-20, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 13.2 μC. (3.0 gm.) C^{14} LABELED ALBUMIN.

hrs. post inj.	a/b	% of "blood" serum albumin from milk derived from blood albumin
2	0.518	---
6	0.675	---
12	0.982	98.2
24	0.995	99.5
48	0.984	98.4
72	0.978	97.8
	0.985 ± 0.00321[1]	

---

[1] The mean value of the ratios from 12 through 72 hours post injection is presented with standard error.

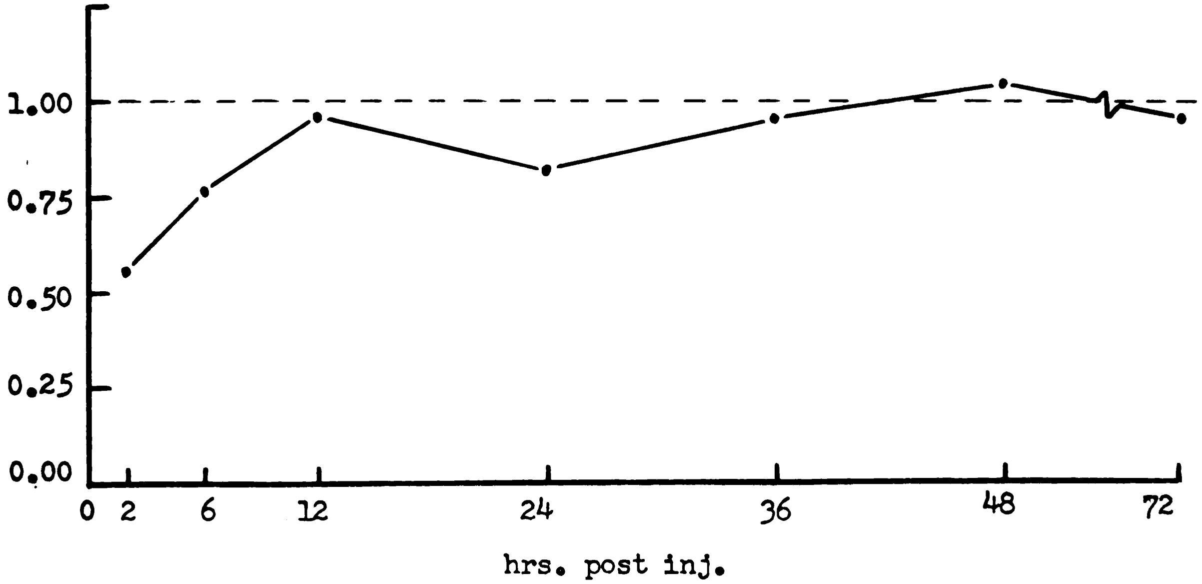

FIGURE 61. RATIOS OF THE SPECIFIC ACTIVITIES OF "BLOOD" SERUM ALBUMINS FROM MILK TO THE SPECIFIC ACTIVITIES OF BLOOD ALBUMIN FROM X-17 (LACTATING RABBIT 10TH-13TH DAYS POSTPARTUM INJECTED WITH 5.16 µC. OF C^{14} LABELED ALBUMIN).

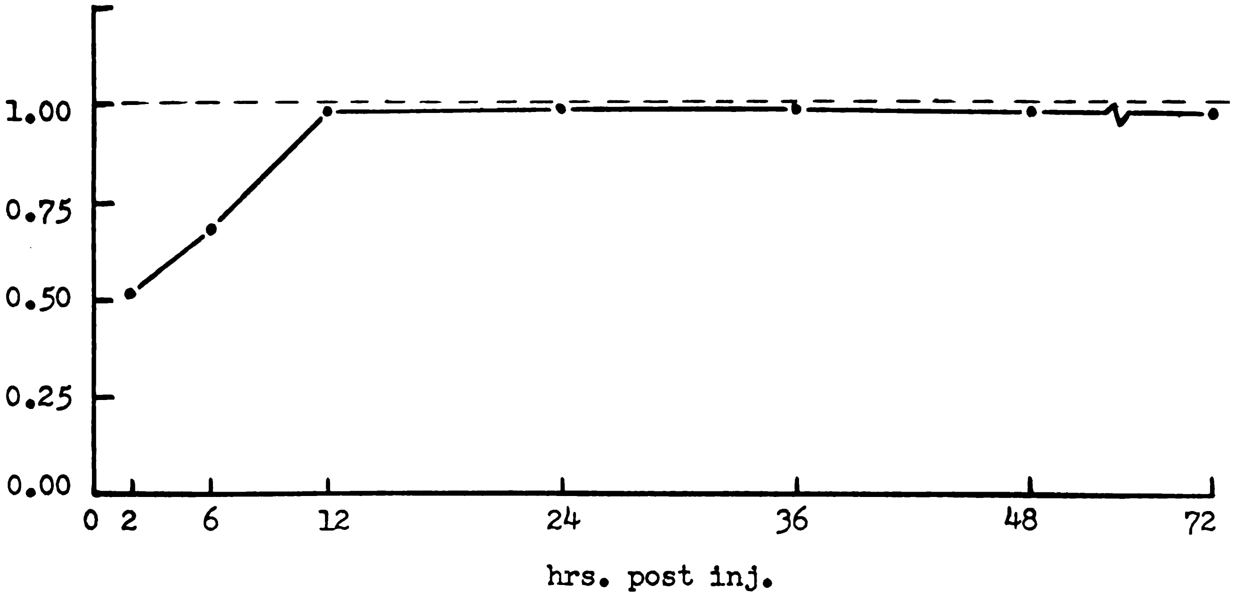

FIGURE 62. RATIOS OF THE SPECIFIC ACTIVITIES OF "BLOOD" SERUM ALBUMINS FROM MILK TO THE SPECIFIC ACTIVITIES OF BLOOD ALBUMIN FROM X-20 (LACTATING RABBIT 10TH-13TH DAYS POSTPARTUM INJECTED WITH 13.2 μC. OF C^{14} LABELED ALBUMIN).

There have been many reports on turnover rate and biological half-times of serum albumins in various species. Dixon et al. (1953) and Cohen et al. (1956) have reported half-time values for radioactive albumins injected into rabbits as 5.7 and 3.8 days, respectively. These reported values agree fairly well with the half-times determined by inspection of the specific activity curves for blood albumins presented in Figures 59 and 69, which are approximately 3 days.

These data presented in Tables 17 through 20 are interesting, since they provide new information concerning albumin in rabbit milk. "Blood" serum albumin in rabbit milk has not been reported in the literature. This is the first work on the injection of C^{14}-albumin into a lactating animal.

E. Experiments 9 and 10. Experiments Designed to Evaluate the Potential of α-Globulins and β-Globulins as Precursors of Rabbit Milk Proteins

The possibility of α- and β-globulins as precursors of milk proteins produced by the rabbit was also investigated. The data for these experiments are presented in Tables 10 and 11 in the appendix. All of the blood and milk protein fractions mentioned previously were isolated and the specific activity of these fractions determined. It can be seen that the activities in all of these fractions were much lower than the activity in the injected fraction. On the basis of these data, it may be concluded that α- and β-blood globulins do not serve as direct precursors of milk proteins nor are any of the milk proteins isolated derived directly from them. The activity which does appear is attributed to amino acids liberated in the body from the catabolism of these radioactive injected fractions.

These data do not preclude the possibility of milk proteins which are derived directly from α- and β-globulins. From the conclusions drawn from the work done on albumins, it seems reasonable to postulate that all the proteins of blood plasma are present in minute quantities in milk. However, it is felt that they are present in such minute quantities that present qualitative methods are not sensitive enough to detect them. This is not unreasonable when the protein hormones are considered. It is certain that they are present in the blood but they have not been chemically isolated and characterized. It would be even more difficult to detect the minor blood proteins in milk since they do not have the physiological activity of the various hormones.

II. Studies of the Hormonal Requirements of Guinea Pig Mammary Tissue *In Vitro*

A. Results

1. Development of Tissue Culture Mediums

The original plan for the tissue culture work contained three basic ideas: (1) to develop a medium capable of maintaining mammary tissue *in vitro*, (2) to develop a medium capable of initiating secretion in non-secretory mammary tissue *in vitro* and (3) to develop a medium capable of maintaining secretion of mammary tissue *in vitro*.

Two of these aims have been accomplished. Media have been developed that will maintain non-secretory and secretory mammary tissue from guinea pigs *in vitro*. Tables 21 and 22 present the media developed. Table 23 presents a medium prepared as a control to demonstrate the effects of the hormones added to the medium. Parker's 199 synthetic medium is a completely synthetic solution composed of 81 nutrients. It is composed of all the known essentials in proper proportions for cell nutrition, containing all of the amino acids, salts, vitamins, and carbohydrates shown to be necessary for cellular maintenance and mitosis. This medium was developed by Morgan *et al.* (1950).

The final concentrations of hormones added to the basic medium are reported in Tables 21 and 22. These are reported in ug./ml. The hydrocortisone used was from Merck, Sharp, and Dohme (Lot No. 28C 0502D) and the prolactin was Panlitar (list 806) Ovine Lactogenic Hormone from Armour Laboratories, Chicago, Illinois. It contained 20 I.U./mg. The insulin preparation used was the crystalline Zn-insulin preparation of Eli Lilly and Co. This preparation contained 25 units of activity per mg. Penicillin was added as a bacteriostatic. It has been known for a long time that serum added to a tissue culture medium is very beneficial

TABLE 21. COMPOSITION OF MEDIUM I DEVELOPED FOR MAINTENANCE OF GUINEA PIG MAMMARY TISSUE *IN VITRO*.

Parker's 199 synthetic medium, adjusted to pH 7.4 with 1.4% of $NaHCO_3$

materials added	concentration
hydrocortisone	8 ug./ml.
prolactin	140 ug./ml.
insulin	70 ug./ml.
penicillin G	50 I.U./ml.
rabbit serum	10%

medium gassed with 95% O_2 and 5% CO_2

TABLE 22. COMPOSITION OF MEDIUM II FOR MAINTENANCE OF ACTIVE SECRETION OF GUINEA PIG MAMMARY TISSUE *IN VITRO*.

Parker's 199 synthetic medium, adjusted to pH 7.4 with 1.4% $NaHCO_3$

materials added	concentration
hydrocortisone	16 ug./ml.
prolactin	240 ug./ml.
insulin	140 ug./ml.
penicillin G	50 I.U./ml.
rabbit serum	10%

medium gassed with 95% O_2 and 5% CO_2

TABLE 23. COMPOSITION OF CONTROL MEDIUM FOR GUINEA PIG MAMMARY TISSUE *IN VITRO*.

Parker's 199 synthetic medium, adjusted to pH 7.4 with 1.4% $NaHCO_3$

materials added	concentration
penicillin G	50 I.U./ml.
rabbit serum	10%

medium gassed with 95% O_2 and 5% CO_2

to the cell and has become a routine procedure in tissue culture laboratories.

The control medium reported in Table 23 was prepared specifically for the purpose of demonstrating the difference between tissues cultured in the presence or absence of the hormones. It was prepared in the same manner as the first 2 media described.

All the media were adjusted to pH 7.4 with sterile 1.4% $NaHCO_3$. The 199 medium contains methyl red. The $NaHCO_3$ is added a drop at a time and the red color developed is compared with a standard methyl red solution. This method causes a slight variation in pH, but this does not seem to be significant. The three media were usually prepared in 50 ml. volumes. Two weeks was the maximum time period that these media were kept. After this length of time, the media were discarded as a precautionary measure to prevent contamination and variations in the media.

2. Results of Explants Prepared From Non-Secretory Guinea Pig Mammary Tissue and Cultured in Medium I

Table 24 presents a summary of the reactions of tissues taken from mammary tissue in a non-secretory state and cultured for 5 days in medium I. The average data presented at the bottom of Table 24 summarize the results of all the cultures on non-secretory mammary tissue from guinea pigs. The tissues were taken from 8 animals. From these 8 animals, a total of 108 explants were prepared and cultured in medium I. Histological examination of these explants revealed that approximately 73% of the total lobulo-alveolar tissue was maintained. The secretory alveoli amounted to only 4.7% of the total alveoli in these explants. This is not considered to be significant due to the crude

TABLE 24. RESULTS OF EXPLANTS PREPARED FROM NON-SECRETORY GUINEA PIG MAMMARY TISSUE AND CULTURED IN MEDIUM I FOR FIVE DAYS.

Animal providing explt.[2]	No. explt.	% of total LA[1] MT[3]	% of total LA secretory	Comments
X-4	12	100.0	0.00	LA appeared like the FTC[4].
X-7	12	25.0	0.00	Most of the explts. were fatty tissue. The LA of FTC appeared to be involuted.
X-8	12	87.5	0.00	LA appeared like the FTC.
X-9	12	70.0	6.65	Very slight secretion in some peripheral LA.
X-13	12	95.0	0.00	LA MT like the FTC.
X-15	12	75.0	18.75	Excellent MT of 9 explt. Three were completely degenerate. Secretion in periphery.
X-16	12	62.5	0.00	Degenerate LA in center of 9 explants. Three explants had enlarged LA.
X-33	24	87.5	8.75	Degenerate LA in center of 6 explants. Secretory LA in periphery of 9 explants.
AVS.	108	73.6	4.70	MT LA appeared similar to FTC. The degenerate LA was usually in center of explt. The secretory LA was at periphery of explt.

[1] LA = lobulo-alveolar tissue

[2] Explt. = explants

[3] MT = maintained

[4] FTC = Fresh tissue control

methods for evaluating the percentage of alveoli that are maintained or secretory.

The data presented in Table 24 provide information about the individual experiments with tissues taken from individual animals. It can be seen that there is considerable variation from one experiment to the next. The percent of lobulo-alveolar tissue maintained varies from 100 to 25%. This extreme variation is noted in explants from animal number X-4, which was evaluated as 100% of the lobulo-alveolar tissue maintained, and explants from animal number X-7, which was evaluated as only 25% of the lobulo-alveolar tissue maintained. The results of the experiments on the explants from the last 6 animals are more consistent. In these 6 experiments, the percent of lobulo-alveolar tissue maintained varies from 62.5% to 95% of the lobular-alveolar tissue present in the tissue.

The comments in the last column of Table 24 give some insight into the morphological condition of the tissue of these explants. Animal X-7, which provided explants that had the lowest percentage of lobulo-alveolar tissue maintained, had mammary tissue which was in a state of involution. This condition might have influenced the _in vitro_ maintenance of the alveoli. An interesting point is illustrated in the behavior of the explants prepared from animal X-15. In this case, 3 of the explants were completely degenerate while the other 9 explants were completely maintained. This is not an uncommon experience in tissue culture.

Some general statements can be made concerning the _in vitro_ maintenance of guinea pig mammary tissue reported in Table 24. The lobulo-alveolar tissue maintained appeared very similar to the lobulo-alveolar tissue of the control tissue taken at the same time that the explants were prepared. Where degeneration of the alveoli occurred, it usually appeared within the center and not in the periphery of the explant. Also, secretory alveoli in the explants cultured in medium I were invariably found in the periphery of the explant, in direct contact with the nutrient medium.

Figure 63 is a photomicrograph of a non-secretory control guinea pig mammary tissue. There is no secretion evident in this tissue. The alveoli are small. The epithelial cells do not show typical secretory vacuoles and secretory granuoles are not prominent in the cytoplasm. Figure 64 is a photomicrograph of an explant taken from the same animal at the same time and cultured in medium I for 5 days. The histological picture is the same as the histological picture of the control. These two photomicrographs demonstrate the ability of medium I to maintain guinea pig non-secretory mammary tissue.

3. Results of Explants Prepared From Secretory Guinea Pig Mammary Tissue and Cultured in Medium II

Table 25 presents a summary of the data on tissues taken from mammary glands in a secretory state and cultured for 5 days in medium II. The data presented at the end of Table 25 summarize the results of the tissue culture experiments done on secretory guinea pig mammary tissue cultured in medium II. In these experiments, 12 animals were utilized to provide secretory mammary tissue with a total of 180 individual explants. Seventy percent of the lobulo-alveolar tissue was maintained in these 180 explants presented in Table 25. This is in remarkable agreement with the percent of lobulo-alveolar tissue maintained in the experiments presented in Table 24. The value given in Table 24 is 73.6% of the lobulo-alveolar tissue maintained. However, Table 25

FIGURE 63. PHOTOMICROGRAPH (130X) OF NON-SECRETORY CONTROL GUINEA PIG MAMMARY TISSUE SECTION STAINED WITH IRON HEMATOXYLIN AND EOSIN.

FIGURE 64. PHOTOMICROGRAPH (130X) OF A SECTION OF AN EXPLANT PREPARED FROM THE SAME ANIMAL AS THE CONTROL TISSUE (FIGURE 63) AND CULTURED IN MEDIUM I FOR FIVE DAYS.

TABLE 25. RESULTS FROM EXPLANTS PREPARED FROM SECRETORY GUINEA PIG MAMMARY TISSUE AND CULTURED IN MEDIUM II FOR FIVE DAYS.

Animal providing explt.[1]	No. explt.	% of total LA[2] MT[3]	% of total LA secretory	Comments
X-5	12	100.00	100.0	Secretion same as FTC[4].
X-6	12	8.75	0.0	Most LA degenerate, tissue disorganized. ½ Explt. contaminated.
X-10	12	0.00	0.0	All explt. degenerate. All contaminated.
X-14	12	50.00	0.0	No secretion in MT LA.
X-17	12	46.00	46.0	Secretion same as FTC.
X-21	12	75.00	10.0	Secretion same as FTC.
X-22	12	75.00	75.0	Heavy secretion in MT LA. Degeneration in center of 6 explt.
X-23	24	75.00	75.0	Secretion same as FTC.
X-25	24	100.00	100.0	Secretion greater than FTC.
X-26	24	73.00	31.3	All cells highly stimulated and vacuolated. Nuclei greater than FTC. FTC. very slightly secretory.
X-28	12	100.00	87.5	All cells highly stimulated and vacuolated. Heavy secretion in secretory LA. No secretion in FTC.
X-29	12	100.00	100.0	All cells highly stimulated and vacuolated. All LA shows good secretion. Very little secretion in FTC.
AVS.	180	70.00	55.4	Secretion in LA of explt. as good or greater than FTC. Cells consistently show enlarged nuclei and secretory vacuoles in the cytoplasm.

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[1] Explt. = explants

[2] LA = lobulo-alveolar tissue

[3] MT = maintained

[4] FTC = fresh tissue control

presents a value of 55.4% for the lobulo-alveolar tissue which was secretory. This is significantly greater than the value of 4.7% reported in Table 24.

Two of the experiments reported in Table 25 were faulty because of contamination. It is felt that this is the reason that the explants from these two experiments showed extensive degeneration and no secretory activity. If the values for these two experiments are discarded, the percentage of secretory lobulo-alveolar tissue maintained would have been higher. If these values are discarded, the average values at the end of Table 25 would be 80.3% of the total lobulo-alveolar tissue maintained and 64.0% of the total lobulo-alveolar tissue secretory. The values presented in Table 25 show a large variation from one experiment to the next. The lobulo-alveolar tissue in explants prepared from animal X-17 was 46% maintained while that from X-5, X-25, X-28 and X-29 appeared to be 100% maintained. The same variation is evident in the secretory lobulo-alveolar tissue. The lobulo-alveolar tissue in explants prepared from X-21 was only 10% secretory while the lobulo-alveolar tissue in explants prepared from X-5, X-25 and X-29 appeared to be 100% secretory.

It is indicated in the last column of Table 25 that in those cultures that were contaminated by bacteria (X-6 and X-10), extensive degeneration of the explants took place. At the end of Table 25 the morphological condition of the explants is summarized. It is apparent that in most of the explants in which secretion was noted, the secretion was as great or greater than in the control of the fresh tissue taken at the time the explants were prepared. Also, the nuclei of the epithelial cells appear to be enlarged when compared to the controls. In those alveoli showing a heavy secretion in the lumen, the epithelial cells lining it appear to have large secretory vacuoles in the cytoplasm.

Figure 65 is a photomicrograph of secretory guinea pig control tissue. The secretion seen in this tissue is of a very low order of magnitude. Secretory mammary tissue was obtained from guinea pigs on the 4th to 6th days postpartum. They were kept with their litters and the litters were allowed to suckle from birth until the sows were sacrificed to provide explants. All secretory control tissues had this appearance. It is difficult to explain this low level of secretory activity. It is possible that the litters did not suckle. The newborn guinea pig is not dependent on the mother's milk, and can survive well on dry feed.

Figure 66 is a photomicrograph of an explant taken from the same animal as the control in Figure 65 and cultured in medium II for 5 days. There is an obvious difference in the appearance of these two tissues from the same animal. The explant shows considerably more secretory activity than the control. The alveoli are enlarged and secretory materials are evident in the alveolar lumina. The nuclei of the epithelial cells are enlarged and secretory vacuoles are very evident in the cytoplasm. The large duct on the left side of the photomicrograph appears to be filled with secretion. It is evident by comparison of Figures 65 and 66 that the secretory activity of this tissue was maintained and likely increased by medium II.

Figure 67 is a photomicrograph of an explant from the same animal as the control tissue in Figure 65. It was cultured in the control medium (Table 23) for 5 days. This photomicrograph is a dramatic demonstration of the hormonal requirements of guinea pig mammary tissue _in vitro_. This explant is completely degenerate. There do not appear to be any viable cells in this explant. It cannot be recognized as mammary tissue.

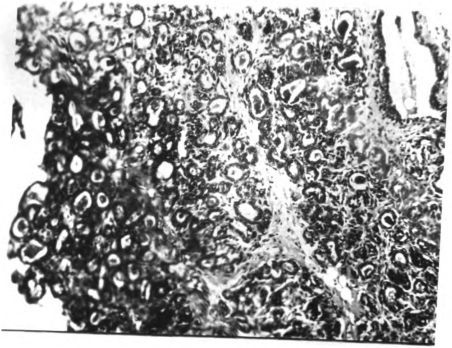

FIGURE 65. PHOTOMICROGRAPH (130X) OF A SECRETORY CONTROL MAMMARY TISSUE TAKEN FROM A LACTATING GUINEA PIG. STAINED WITH HEMATOXYLIN AND EOSIN.

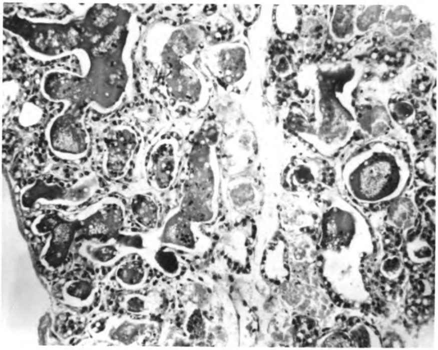

FIGURE 66. PHOTOMICROGRAPH (130X) OF A SECTION OF AN EXPLANT TAKEN FROM THE SAME TISSUE SHOWN IN FIGURE 65 AND CULTURED FOR FIVE DAYS IN MEDIUM II.

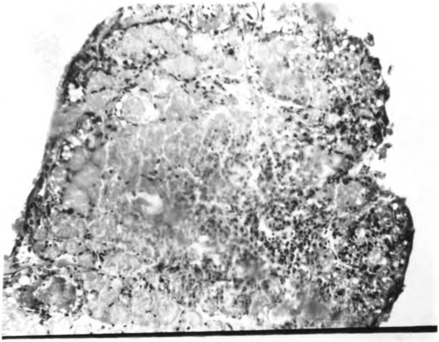

FIGURE 67. PHOTOMICROGRAPH (130X) OF A SECTION OF AN EXPLANT PREPARED FROM THE SAME TISSUE AS SHOWN IN FIGURE 65 AND CULTURED FOR FIVE DAYS IN THE CONTROL MEDIUM.

4. Results of Explants Prepared From Secretory Guinea Pig Mammary Tissue and Cultured in Medium II Containing DL-Leucine-2-C^{14} or DL-Glutamic Acid-2-C^{14}

There is a possibility that the apparent secretion observed in the photomicrograph in Figure 66 is an artifact and not the accumulation of true secretory products. In order to establish that the mammary tissue explants were actually taking up materials from the medium and secreting them into the alveoli via the epithelial cells, 0.2 µc. of either DL-leucine-2-C^{14} or DL-glutamic acid-2-C^{14} was added to medium II. Figures 68 and 69 are photomicrographs of autoradiographs prepared from explants cultured in radioactive mediums. Figure 68 is from an explant cultured in a medium containing the labeled glutamic acid and Figure 69 is from an explant cultured in a medium containing the labeled leucine. It may be seen from these autoradiographs that these labeled amino acids were taken up and concentrated in certain areas of the tissue.

Photomicrographs of autoradiographs are difficult to prepare. In order to show the exposed developed emulsion, the optical system of the microscope must be focused on the emulsion spread over the tissue. Because of this, the tissue is out of focus. When the slide is placed under the microscope, it is possible to focus on the emulsion and then refocus the same field on the stained tissue. By this technique, it was possible to determine that the areas of concentrated radioactivity were located directly over highly secretory alveoli. This provides further evidence that these explants cultured in medium II were able to take up these radioactive amino acids and concentrate them in the areas of active secretion. It seems reasonable to expect that these amino acids were being incorporated into mammary secretory materials. One autoradiograph showed exposed emulsion along the entire length of a duct which was cut in longitudinal section. Unfortunately, the degree

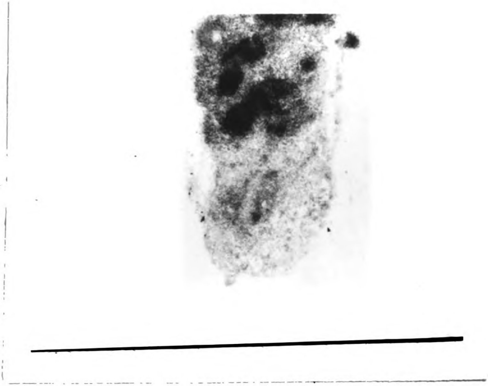

FIGURE 68. PHOTOMICROGRAPH OF AN AUTORADIOGRAPH PREPARED FROM AN EXPLANT CULTURED FOR FIVE DAYS IN MEDIUM II CONTAINING 0.2 μc./ml. OF DL-GLUTAMIC ACID-2-C^{14}.

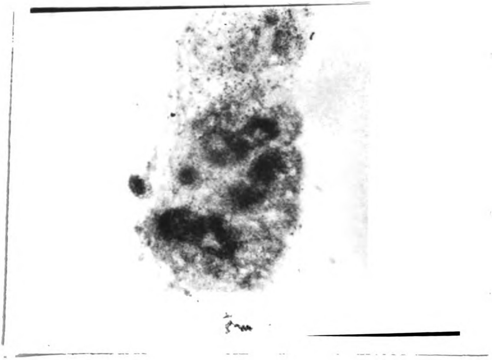

FIGURE 69. PHOTOMICROGRAPH OF AN AUTORADIOGRAPH PREPARED FROM AN EXPLANT CULTURED FOR FIVE DAYS IN MEDIUM II CONTAINING 0.2 μc./ml. OF DL-LEUCINE-2-C^{14}.

of exposure was so light that it was impossible to take a photograph of this autoradiograph. This indicates that the secretory explants not only take up materials and accumulate them in areas of active secretion but can also transfer them to the duct system.

5. Results of Explants Prepared From Secretory Guinea Pig Mammary Tissue and Cultured in Medium II Containing C^{14}-γ-Globulin or C^{14} Albumin

Due to the interesting results obtained on the γ-globulins and albumins reported in the work on the injection of labeled compounds into lactating rabbits, it was decided to try to demonstrate incorporation of radioactive γ-globulin and albumin into secretory mammary tissue explants *in vitro*. Medium II was prepared and C^{14}-γ-globulins and C^{14}-albumin were added to portions of it to give an activity of 0.1 μc./ml. of medium. Explants from the same lactating animal were cultured in these mediums. Also, explants from this animal were placed in sterile water at 50°C for one minute and then cultured in the same mediums. This heat treatment of explants was also carried out on explants cultured in the radioactive leucine and glutamic acid mediums.

Figure 70 is a photomicrograph of a autoradiograph prepared from an explant cultured in the medium containing C^{14}-γ-globulin (0.1 μc./ml. of medium). This photomicrograph shows quite clearly that the activity of the C^{14}-γ-globulin is concentrated in the secretory alveolar cells. This experiment was duplicated on explants from another lactating guinea pig. This has been done on a total of 16 explants from two animals. The explants cultured in the medium containing C^{14} albumin (0.1 μc./ml. of medium) did not show any exposure of the emulsion, although they were secretory upon histological examination of the explants. Also, the heat treated explants showed no exposure of the emulsion in any of the

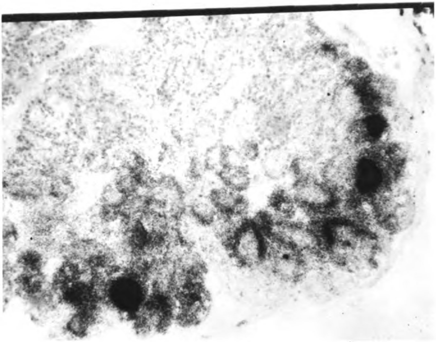

FIGURE 70. PHOTOMICROGRAPH OF AN AUTORADIOGRAPH PREPARED FROM AN EXPLANT CULTURED FOR FIVE DAYS IN MEDIUM II CONTAINING 0.1 μc./ml. OF C^{14} LABELED γ-GLOBULIN.

experiments with leucine-2-C^{14}, glutamic acid-2-C^{14}, C^{14}-γ-globulin, or C^{14}-albumin. On the basis of these results, it may be concluded that γ-globulin can be taken up and utilized by actively secreting mammary tissue. Also, that albumin is not taken up to any major extent by these explants *in vitro*.

B. Discussion

1. Preliminary Studies on the Cultivation of Rat, Mouse and Rabbit Mammary Tissue *In Vitro* in Which Negative Results Were Obtained

There are many difficulties encountered in tissue culture. Some of these have been described previously in the methods section, such as preparation of glassware, mediums, explants, and bacterial contamination. Over the years, mammary tissue has been one of the most difficult tissues to culture. Lasfargues (1957a) reported the successful maintenance and proliferation of mammary epithelium removed from adult C-57 strain mice prior to the tenth day of pregnancy. The epithelial cells were dispersed by incubation with collagenase for 1 to 2 hours. The free cells were then cultured in various mediums. He reported that serum from human placenta gave the best results, and noted a proliferation of sheets of epithelial cells. He states that these sheets of epithelial cells were organized into a typical mammary pattern. However, the photomicrographs in his paper do not appear to have a typical organization when compared to a histological preparation of mouse mammary tissue.

Elias (1957) reported that organ cultures of mammary tissue taken from C3H/He CR GL strain mice could be maintained by means of synthetic "199" medium enriched with hormones. He further showed that it was possible to stimulate very slight secretion in these explants with his high level medium. He enriched his medium with estrone, progesterone, cortisol, growth hormone, and mammotropic hormone in two concentrations. The high concentration contained 0.2, 2.0, 8.0, 140, and 140 ug./ml. of medium, respectively. The low concentration contained 0.02, 2.0, 2.0, 20, and 20 ug./ml. of medium, respectively. These mediums of Elias (1957) provided the basis for the mediums ultimately developed for the guinea pig (Tables 21 and 22).

An attempt was made to duplicate the work of Elias (1957) on mice. In this study, C-57 strain mice, albino rats, and New Zeeland white rabbits during mid-pregnancy or a few days before parturition, and during various stages of lactation were used. The mediums and methods of culture were identical with those of Elias (1957). This work was completely unsuccessful. There are a number of possible explanations for the failure of these experiments. It is to a certain degree surprising that Elias (1957) has been able to initiate lactation *in vitro* in the presence of estrogen and progesterone. Nelson (1936) has postulated that estrogen has a direct inhibitory effect on the lactational performance of the mammary gland. Meites and Turner (1942a,b,c) have postulated that there are stimulatory as well as inhibitory factors involved in the initiation of lactation. They have shown that estrogen in the intact animal can significantly increase the pituitary prolactin content and that the level of prolactin in the blood of the rabbit is increased. It is possible that estrogen at some doses has an inhibitory effect on milk secretion as postulated by Nelson (1936). There is no doubt about the effect of prolactin on mammary tissue.

The results of Elias (1957) might be explained by the fact that the concentration of prolactin was high enough to overcome any inhibitory effects of estrogen in the medium. This is compatible with the theories of Meites and Turner (1942a,b,c) and with the results of

Sgouris and Meites (1954, 1955) on the rabbit. The failure to obtain positive results with the mediums of Elias (1957) on mice, rats, and rabbits might be explained by the fact that the concentrations of estrogen and progesterone were too high to be overcome by the level of prolactin added to the mediums. The prolactin used by Elias (1957) was supplied by Dr. C. H. Li of the University of California, Berkeley (Li preparation L2738E). It is possible that Li's preparation is much purer than Armor's Fanlitar, used in this work, and thus contained more prolactin per unit weight of material.

There are other factors which might have contributed to the failure of these initial trials. It is possible that there are species differences involved. It is also possible that there are slightly different requirements of the absolute levels of hormones necessary to maintain various types of mammary tissues and also the ratios of these hormones might vary slightly. Improper preparation of glassware might have caused a toxicity which was not apparent to the investigator during this early work. Also, this work was the first attempt in the area of tissue culture. It seems reasonable to believe that inexperience might also have contributed to the lack of positive results in this early work.

2. Development of Mediums for the Maintenance of Secretory and Non-Secretory Guinea Pig Mammary Tissue Explants *In Vitro*

The early difficulties encountered in tissue culture experiments prompted trials with various combinations, levels, and ratios of hormones. Lyons *et al.* (1958) have shown conclusively that prolactin and corticoids are essential for the initiation of lactation in the fully developed mammary glands of hypophysectomized, oophorectomized, adrenalectomized Long-Evans rats. Elias (1957) also reported that he was able to maintain his cultures with prolactin and cortisol alone. Elias (1959) and Trowell (1959) reported that insulin had a beneficial effect on mammary tissue explants and that it alone was capable of maintaining the tissue to some extent. Trowell (1959) also reported that a high oxygen gas phase was beneficial in the maintenance of rat mammary explants. Lasfargues (1957a,b) described the proliferation of sheets of mammary epithelium grown in various types of serum.

On the basis of these reports of mammary tissues grown in a variety of media and on the basis of Lyons' <u>et al</u>. (1958) results, it was decided to prepare the medium described in Table 21. This medium was first used to test the maintenance of rabbit mammary tissue <u>in vitro</u>. There was very minimal maintenance of these explants. The alveoli were degenerate but there were very normal viable epithelial cells in the explants. In some respects this was discouraging but not completely so. A decision was made to attempt the cultivation of guinea pig mammary tissue. This was done first with mammary tissue from a non-lactating guinea pig. Excellent maintenance of this tissue was achieved.

Various other modifications of this medium were then tried to improve it with the ultimate aim of initiation of lactation <u>in vitro</u>. The level of hydrocortisone was doubled. This caused harmful effects in that the epithelial cells were maintained but the alveolar organization was destroyed. This finding that high levels of hydrocortisone can be harmful has been confirmed by Larson (1959) in his work with the cultivation of bovine mammary epithelium. Higher levels of prolactin and insulin did not appear to be of additional benefit for the maintenance of non-secretory guinea pig mammary tissue. Medium I without rabbit serum will maintain non-secretory guinea pig mammary tissue. However, the explants do not look as good as those cultured in the presence of the rabbit serum. Growth hormone did not appear to have any beneficial effects on the maintenance of these non-secretory explants. This work has provided a medium for good maintenance of non-secretory guinea pig mammary tissue but it has not resulted in the initiation of lactation *in vitro*.

Next, mammary tissue from a lactating guinea pig was cultured in medium I. It was surprising to find that the explants from this tissue were completely degenerate. This indicated that the requirements for secretory mammary tissue were different than the requirements for non-secretory mammary tissue. From a biochemical point of view, it might be expected that the nutritional requirements would be different. The levels of hydrocortisone, prolactin, and insulin were doubled over the levels reported in Table 21 to prepare medium II described in Table 22. Explants prepared from secretory guinea pig mammary tissues were cultured in medium II. At the end of the culture period, it was found upon histological examination that the tissue was highly secretory. The secretion was as good or better than the secretion found in the non-cultured control. Again, variations of this medium were prepared and evaluated. None of these variations maintained the secretory tissue any better than the medium reported in Table 22.

Explants were prepared from non-secretory guinea pig mammary tissue and cultured in medium II. The aim was to initiate lactation in this non-secretory mammary tissue *in vitro*. The explants from this non-secretory mammary tissue were well maintained, but there was no initiation of secretion in these explants.

The serum used in these media was drawn from healthy male rabbits. It is doubtful that the endogenous hormones of this serum had any beneficial effect on the cultures. The serum was added in the same concentration (10%) to the control medium as well as mediums I and II. All tissues cultured in the control medium (without hormonal additions) were completely degenerate upon histological examination.

These results on the development of adequate mediums for the maintenance of non-secretory and secretory guinea pig mammary tissue *in vitro* show conclusively that the hormonal requirements of secretory and non-secretory guinea pig mammary tissues *in vitro* are different. The requirements for prolactin, hydrocortisone, and insulin are two times as high for the maintenance of secretory tissue as they are for the maintenance of non-secretory tissue. Unfortunately, there are no other reports on the cultivation and maintenance of secretory mammary tissue by the organ culture technique in the guinea pig. Thus, no direct comparisons can be made with the work of others.

The hormonal requirements of guinea pig mammary tissue *in vitro* are in agreement with the hormonal requirements reported for bovine mammary epithelium by Larson (1959), Ebner and Larson (1958, 1959), and Hoover *et al.* (1959). The hormonal requirements are also in agreement with those reported by Elias (1957, 1959) and Elias and Rivera (1959) for mouse mammary. The absolute amounts of the hormones required are difficult to compare in the case of prolactin since the activities of Li's preparations used by Elias (1957) are not reported. However, the quantity of insulin used by Elias and the quantity in medium I (Table 21) are the same. Also, Elias (1957) used 8 µg./ml. of cortisol and medium I contained the same amount.

Although a medium was not developed which would initiate secretion in a non-secretory mammary tissue, the results are encouraging. Table 25 presents data which show that the secretory activity of some of the explants could be enhanced by medium II. Thus, there is some evidence for a hormonal stimulus increasing the lactational performance of guinea pig mammary tissue explants *in vitro*. On the basis of these data, it is felt that the initiation of lactation for the purpose of evaluating hormonal requirements *in vitro* in guinea pig mammary tissue can be accomplished. It is believed that the right combination or ratio of prolactin, hydrocortisone, and insulin would be effective. This is a very extensive task when all of the possible combinations are considered.

It is interesting to draw some general comparisons between the levels of prolactin used in medium II and levels of prolactin that will produce a response *in vivo*. Meites *et al.* (1941) demonstrated a response in the crop sacs of 50% of pigeons injected intradermally over the crop sacs with a total of 0.00624 I.U. of prolactin. Medium II contained 4.8 I.U. of prolactin per ml. This is a much higher level than Meites (1941) has shown necessary for pigeon crop proliferation. Meites and Turner (1950) have shown that the pituitaries of various mammals contain from 1.11 to 29.5 I.U. of prolactin per mg. of pituitary tissue. On the basis of these data, it appears that mammary tissue *in vitro* requires much more prolactin than mammary tissue *in vivo*. It should be pointed out that the explant is dependent primarily upon diffusion of substances from the medium into the tissue. This might in part explain the apparently large difference between the *in vivo* and *in vitro* requirements.

The photomicrographs demonstrate clearly the hormonal dependence of guinea pig mammary tissue in vitro. They also demonstrate the differences in the hormonal requirements of secretory and non-secretory mammary tissue. On the basis of the work with secretory mammary tissue, it appears that the level of secretion within mammary tissue is not as important as whether it is secretory or non-secretory. As long as there is some secretion, it appears that it is possible to increase the rate of secretion in vitro by means of prolactin, hydrocortisone, and insulin.

The finding that guinea pig mammary tissue can be maintained in a state of active secretion is important. It provides a system for long term biochemical studies on the lactating mammary gland isolated from the influences of the rest of the body. The need for metabolically active tissues isolated from the hormonal influences of the organism and from other tissues and the organism as a whole has been discussed by Fell (1954).

The results of these in vitro experiments demonstrate quite clearly the importance of organ cultures in the study of endocrinology and biochemistry of the mammary gland. These studies with isotopes on organ cultures of the mammary gland are very interesting. They have provided some insight into the way in which the mammary gland obtains materials for synthesis of milk constituents. This is the first work with tissue culture which has demonstrated the ability of mammary epithelium to take up and utilize native proteins (γ-globulins).

Heating the explants to 50° centigrade appears to destroy their ability to take up γ-globulins, leucine, and glutamic acid. It seems probable that there are enzyme systems involved in the uptake of these substances by the mammary gland.

It appears as though there is a different mechanism or process within mammary tissue for the uptake of γ-globulins and albumins and their ultimate appearance in milk. This *in vitro* work described above lends support to the discussion of the mechanisms involved in the transport of γ-globulins and albumins from the blood to the milk at the end of the section on the results of the injection of radioactive materials into lactating rabbits.

Studies on the organ culture of mammary tissue are relatively new. As demonstrated by this work, the organ culture of mammary tissue can extend our knowledge of the endocrinology, biochemistry, and physiology of the mammary gland.

SUMMARY

1. The objectives of this research were:

a. To provide quantitative data on the precursors of caseins, β-lactoglobulin and α-lactoglobulin of rabbit milk.

b. To provide quantitative data on the relationship between γ-globulins of blood and immune globulins of rabbit milk.

c. To provide quantitative data on the relationship between albumin of blood and the "blood" albumin of rabbit milk.

d. To develop a synthetic medium enriched with hormones which would be capable of maintaining non-secretory mammary tissue _in vitro_.

e. To develop a medium enriched with hormones capable of initiation of lactation in non-secretory mammary tissue _in vitro_.

f. To develop a medium enriched with hormones capable of maintaining secretion in secretory mammary tissue _in vitro_

g. To study the ability of secretory mammary tissue to take up amino acids and blood proteins _in vitro_.

2. The methods utilized in this research involved the blood fractionation by the procedures of Cohn _et al._ (1950). Albumins, γ-globulins and crude fractions of α-globulins and β-globulins were obtained by this method. Paper electrophoresis patterns were run on these fractions and on whole serum to prove their identity and establish their homogeneity.

Two male New Zeeland white rabbits were injected with C^{14} labeled $BaCO_3$. The animals were placed in a chamber designed and built to trap expired C^{14} labeled CO_2. Six hours post injection, the animals were bled and C^{14} labeled serum protein fractions were isolated by the method of Cohn _et al._ (1950).

Classical ammonium sulfate fractionation procedures were employed for the fractionation of the β-lactoglobulin, α-lactalbumin and "blood" albumin in rabbit milk. Rabbit milk caseins were precipitated by adjusting the pH to their isoelectric point which was found to be 4.3. It was found that the immune globulin fraction of rabbit milk could be isolated by readjustment of the acid whey to pH 6.0. A method for paper electrophoresis of rabbit whey and rabbit milk proteins was developed which gave very good results. This method was the precoating of the paper strips with gelatin, thus preventing the absorption of the whey proteins into the paper strips.

The percent of leucine and glutamic acid were determined in the blood protein and milk protein fractions. This was accomplished by hydrolysis of the proteins and by column chromatography. The eluents from the column were subjected to paper chromatography as a purity check. Aliquots of eluents were treated with ninhydrin and the amount of amino acid determined colorimetrically. Free blood leucine and glutamic acid were isolated from the blood by column chromatography. The chemical purity of DL-leucine-2-C^{14} and DL-glutamic acid-2-C^{14} was established by paper chromatography and subsequent counting of the paper strips in a gas flow strip counter.

A method was developed for the milking of rabbits. This was accomplished by injecting 1.0 I.U. of oxytocin into the marginal ear vein. A beveled glass tube was placed over the nipple immediately following the injection. The tube was connected to a reservoir for collection of milk which was in turn connected to a water aspirator. A negative pressure of 15 mm. of Hg. was maintained in the system. Vigorous massage of the mammary gland from the periphery toward the base of the nipple was applied together with periodic interruption of the suction by partial removal of the tube from the nipple. Blood samples were obtained from the rabbits by heart puncture into the left ventricle.

DL-leucine-2-C^{14}, DL-glutamic acid-2-C^{14} and C^{14}-protein fractions were dissolved in a minimal amount of phosphate buffer (pH 7.2) and injected by marginal ear vein into 10 lactating rabbits. Blood and milk samples were obtained at 2, 6, 12, 24, 36, 48 and 72 hours post injection. The blood and milk samples were fractionated into the various serum and milk protein fractions described above.

The blood and milk protein fractions were dialyzed against distilled water until free of reagents. The fractions were then lyophillized. Glutamic acid was isolated from the protein fractions by acid hydrolysis and precipitation of the chloride of glutamic acid. The protein, free glutamic acid and leucine samples were counted by β-liquid scintillation counting procedures.

Mammary tissue was cultured by the organ culture method. The explants were placed on treated rafts of cellulose-acetate. The rafts were floated in the synthetic medium in a watch glass in a Petri dish. The explants were cultured for 5 days. The medium was changed when the pH dropped below 7.0. Histological sections of the explants were prepared and stained with iron hemotoxylin and eosin in the usual manner.

Media for mammary tissue culture were prepared from Parker's "199" synthetic medium. Prolactin, hydrocortisone and insulin were added at 2 concentration levels. The levels of these hormones for mammary tissue maintenance were 140, 8 and 70 ug./ml. of medium, respectively. The levels of these hormones for maintenance of secretion in mammary tissue were 240, 16 and 140 ug./ml. of medium, respectively. The media were gassed with 95% O_2 and 5% CO_2. DL-leucine-2-C^{14}, DL-glutamic acid-2-C^{14}, C^{14}-γ globulin and C^{14}-albumin were also added to portions of the media for maintenance of secretion in vitro.

Autoradiographs were prepared from explants cultured in media containing C^{14} labeled amino acids or proteins. The sections of the tissues were mounted on microscope slides and stained with hemotoxylin and eosin in the usual way. The slides were coverslipped with celloidin. A thin film of Eastman Kodak emulsion was applied in the darkroom. The slides were sealed in a light proof container and placed in the refrigerator for exposure. The exposed autoradiographs were developed with dektal, fixed in acid fix and washed with tap water. They were then dehydrated and coverslipped in the usual manner.

3. The major results and findings of this work are as follows:

Milk Protein Studies

a. In Experiment 1 in which 45 µc. of DL-leucine-2-C^{14} was injected into a lactating rabbit, the free blood leucine decreased at an extremely rapid rate during the first 10 minutes post injection. The calculated value at zero time was 157.2 µc./mm. in the extracellular water. At 10 minutes, the experimentally determined value was 3.7 µc./mm. The equation for this disappearance of free blood leucine is $A = A_0e^{-0.375t}$. The $t_{\frac{1}{2}}$ value is 1.85 min. and the rate constant is -0.375 min.$^{-1}$. After 10 minutes the rate constant changes very drastically to a value of -0.00169 min.$^{-1}$ with a $t_{\frac{1}{2}}$ of 409.5 min. A system to explain these interesting findings has been postulated. On the basis of these data and the shape of the curve, it has been postulated that the leucine injected has been sequestered somewhere in the animal's body. Likely possibilities are the reticulo-endothelial system, the intracellular pool and plasma protein binding. After two hours the leucine from this sequestered pool appears to return to the extracellular leucine pool at a rate slightly less than the rate of removal of free leucine from the extracellular pool. Thus, it appears that only a small proportion of the injected DL-leucine-2-C^{14} was available for synthesis into milk proteins at any particular time. Thus, an attempt was made to establish the parameters for leucine as a direct precursor of milk proteins. It appears that the curve for free blood leucine as determined in Experiment 1 represents the amount of free blood leucine available for incorporation into milk proteins.

b. The data in Experiment 1 show that the leucine incorporated into caseins, β-lactoglobulin and α-lactalbumin of rabbit milk are derived from the free blood leucine. The curves presented fit the criteria for precursor product relationships very well. Further, it takes an average of 4 hours for a free blood leucine molecule to be incorporated into a milk protein and transferred to the ducts ready for excretion as a milk constituent.

c. The ratios of the specific activities of blood γ-globulins and milk immune globulins in Experiment 1 show that at least 73.5% of the milk immune globulins were derived directly from the blood γ-globulins. The immune globulins of the milk were not synthesized within the mammary gland since the specific activities of leucine incorporated into the immune globulins of milk and the specific activities of caseins, β-lactoglobulins and α-lactalbumins are different.

d. It is also concluded from Experiment 1 that at least 77.4% of the "blood" serum albumin in rabbit milk is derived directly from the albumin of blood plasma. This is based on the ratios of the specific activity in these 2 protein fractions. The blood γ-globulins and albumin rapidly achieve equilibrium with the milk immune globulins and "blood" serum albumin of milk. The ratio of the specific activity of the immune globulin from milk to γ-globulin from blood at 2 hours post injection was 0.916 and the ratio of specific activity of "blood" serum albumin from milk to albumin from blood at 2 hours post injection was 0.900, thus demonstrating the rapid equilibrium between these blood proteins and their corresponding milk proteins.

e. Other experiments with DL-leucine-2-C^{14} and DL-glutamic acid-2-C^{14} completely substantiate the results on the first experiment with DL-leucine-2-C^{14}. It is noteworthy that all of the glutamic acid incorporated into caseins, β -lactoglobulin and α-lactalbumin of the milk of lactating rabbits apparently comes directly from the free blood glutamic acid.

f. Experiments were designed to evaluate the importance of plasma proteins as precursors of milk proteins by the injection of C^{14} labeled -plasma protein fractions into lactating rabbits. When C^{14} labeled γ-globulins were injected into 2 lactating rabbits, a close correlation was obtained between the specific activities of the γ-globulins and milk immune globulins. The time required to establish equilibrium between the blood and the milk fractions was slightly more than 2 hours in one animal and slightly more than 6 hours in the other animal. After equilibrium had been reached, it was calculated from the ratios of the specific activities of milk immune globulins to γ-globulins that 93.4% of the milk immune globulin in the milk of one animal and 98.2% of milk immune globulin in the milk of the other animal were derived from the γ-globulin in the plasmas.

g. When C^{14} labeled albumins were injected into 2 lactating rabbits, the relationship between the plasma albumins and "blood" serum albumin in milk was found to be similar to that for γ-globulins and milk immune globulins. In this case, it took between 6 and 12 hours to establish an equilibrium between the blood plasma albumin and the "blood" serum albumin from milk. This was true in both animals.

After equilibrium had been established, it was calculated from the ratios of the specific activities of "blood" serum in milk to the specific activities of albumin in plasma that 92.2% of the "blood" serum albumin in the milk of one animal and 98.2% of the "blood" serum albumin in the milk of the other animal were derived from the albumin in the plasma.

h. Studies on the injection of C^{14} labeled α- and β-globulins from blood plasma indicated that they were of little importance as precursors of milk proteins. Also, no milk protein fraction was detected which was derived directly from these two blood protein fractions.

<u>In Vitro</u> Studies with Mammary Tissue Cultures

a. It was shown that γ-globulin can be taken up by mammary tissue in active secretion and concentrated in the alveolar cells <u>in vitro</u>. This ability is destroyed by heating the explant to 50°C just prior to cultivation.

b. Prolactin (240 µg./ml.), hydrocortisone (16 µg./ml.) and insulin (140 µg./ml.) added to a synthetic medium are capable of maintaining active secretion in mammary tissue <u>in vitro</u>. This was shown by histological preparations and by the active uptake of leucine and glutamic acid and their apparent incorporation into mammary secretory products.

c. Albumin is not taken up by the secretory mammary tissue <u>in vitro</u>.

d. Heating explants to 50°C for 1 minute destroys the ability of mammary tissues to take up γ-globulins, leucine and glutamic acid *in vitro*.

e. Prolactin (140 ug./ml.), hydrocortisone (8 ug./ml.) and insulin (70 ug./ml.) in a synthetic medium are capable of maintaining non-secretory mammary tissue *in vitro*. Seventy-three percent of the total lobulo-alveolar tissue was maintained in 108 explants prepared from 8 non-lactating guinea pigs.

f. Prolactin (240 ug./ml.), hydrocortisone (16 ug./ml.) and insulin (70 ug./ml.) in a synthetic medium are capable of maintaining secretory mammary tissue *in vitro*. Seventy percent of the total lobulo-alveolar tissue was maintained, and 55.4% of the total lobulo-alveolar system was maintained in an active secretory state in 180 explants prepared from 12 lactating guinea pigs.

g. The data obtained on milk immune globulins and "blood" serum albumin in milk and the data obtained by the addition of C^{14} labeled γ-globulins and albumins to tissue culture media indicate that there is a different mechanism by which the mammary epithelium incorporates these proteins into milk. It is postulated that the mammary gland actively takes up γ-globulins or is permeable to γ-globulins, and that it does not actively take up plasma albumin or that the mammary gland is relatively impermeable to albumin.

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APPENDIX

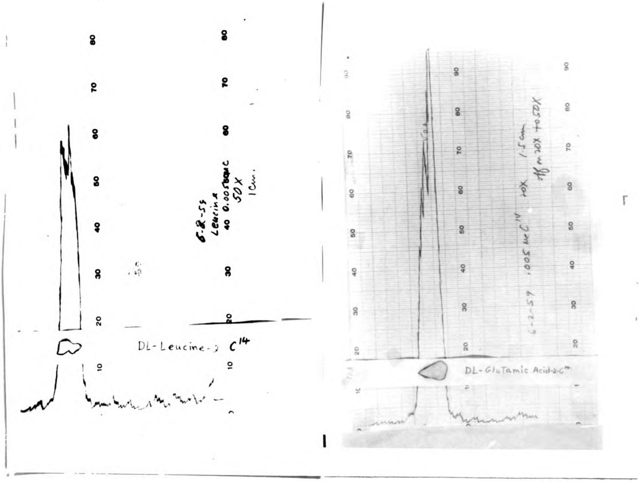

FIGURE 1. PHOTOGRAPHS OF PAPER CHROMATOGRAMS OF DL-LEUCINE-2-C^{14} AND DL-GLUTAMIC ACID-2-C^{14} WITH RADIOACTIVE RECORDINGS.

TABLE 1. THE YIELDS, SPECIFIC ACTIVITIES AND PERCENT OF INJECTED DOSE OF SERUM PROTEIN FRACTIONS ISOLATED FROM MALE RABBITS (X-11 AND X-12) INJECTED WITH 1.50 μC. of $BaCO_3$-C^{14}.

Protein Fraction	mg.	μc./mg.	% Injected Dose of C^{14}
	Rabbit X-11		
γ-Globulins	1,112	0.00033	0.044
Albumin	4,270	0.0026	0.74
α-Globulins	553	0.0029	0.11
β-Globulins	897	0.0021	0.13
	Rabbit X-12		
γ-Globulins	2,045	0.0025	0.33
Albumin	6,312	0.0044	1.85
α-Globulins	1,118	0.0033	0.25
β-Globulins	877	0.0051	0.30

TABLE 2. A SUMMARY OF THE TREATMENTS OF THE LACTATING RABBITS UTILIZED IN EXPERIMENTS 1 THROUGH 10

Exper. No.	An. No.	Wt. Kg.	Days PP[1]	Materials Injected			Samples	
				Material	μc.	mg.	Milk (ml.)	Blood (ml.)
1	X-35	5.0	14-17	L[2]	45.0	11.5	14.6	15.0
2	X-32	5.1	14-17	L	45.0	11.5	15.0	15.0
3	X-30	4.9	14-17	G[3]	150.0	41.2	16.0	15.0
4	X-34	5.2	14-17	G	150.0	41.2	12.5	15.0
5	X-18	5.0	10-13	γ[4]	0.13	400	12.1	15.5
6	X-19	4.9	10-13	γ	2.00	800	11.6	13.3
7	X-17	5.5	10-13	A[5]	5.16	2000	17.5	14.5
8	X-20	4.8	10-13	A	13.20	3000	15.0	13.5
9	X-24	5.2	10-11	α[6]	1.63	492	15.0	15.0
10	X-27	3.7	10-11	β[7]	2.37	398	15.0	15.0

[1] PP = Postpartum

[2] L = DL-Leucine-2-C^{14}

[3] G = DL-Glutamic Acid-2-C^{14}

[4] γ = C^{14} γ-Globulin

[5] A = C^{14} Albumin

[6] α = C^{14} -Globulin

[7] β = C^{14} -Globulin

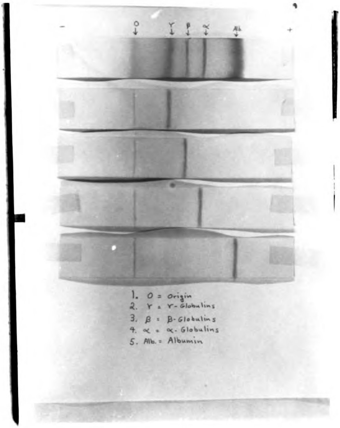

FIGURE 2. PHOTOGRAPH OF PAPER ELECTROPHORESIS PATTERNS OF RABBIT SERUM AND PROTEIN FRACTIONS ISOLATED FROM THE SERUM.

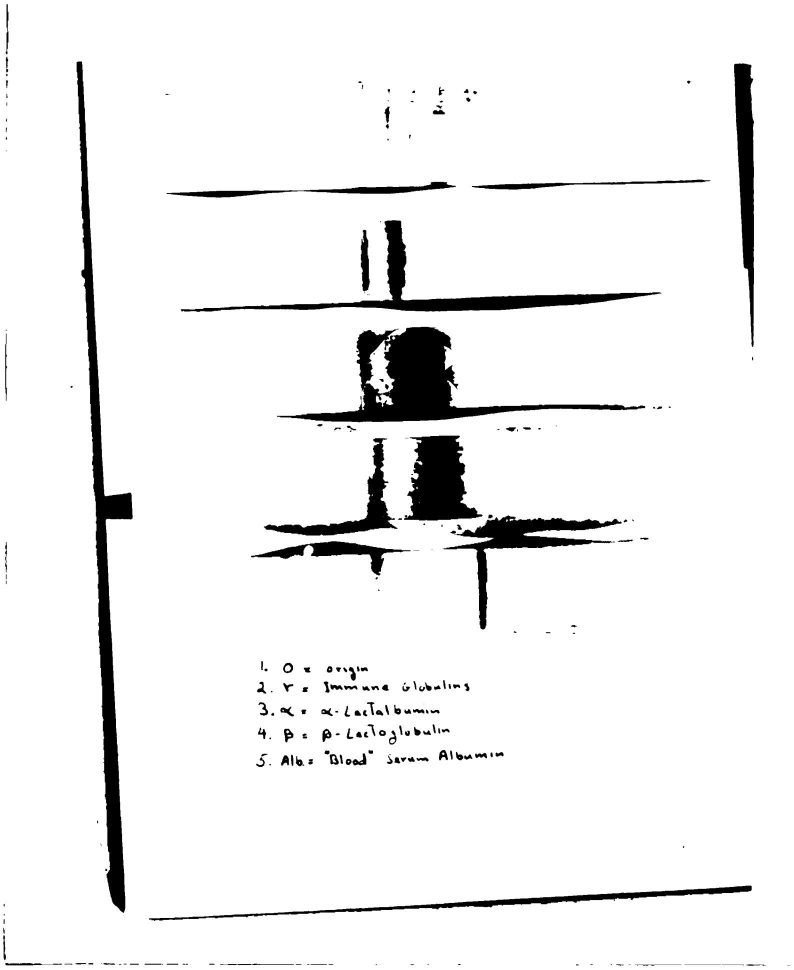

FIGURE 3. PHOTOGRAPH OF PAPER ELECTROPHORESIS PATTERNS OF RABBIT WHEY AND PROTEIN FRACTIONS ISOLATED FROM THE WHEY.

TABLE 3. FREE LEUCINE LEVELS IN THE SERUM OF X-35 (LACTATING RABBIT 13TH-17TH DAYS POSTPARTUM INJECTED WITH 11.5 mg. OF DL-LEUCINE-2-C^{14}).

hrs. post inj.	mg. %
0.16	2.90
2	2.55
6	2.64
12	2.49
24	2.50
48	2.60
72	2.54
AV.	2.60 ± 0.0534[1]

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[1] The mean value with standard error.

TABLE 4. AVERAGE OF TEN VALUES WITH STANDARD ERRORS FOR THE PERCENT OF LEUCINE AND GLUTAMIC ACID IN RABBIT SERUM AND MILK PROTEINS.

Protein	% Leucine	% Glutamic Acid
Albumin	9.5 ± 0.0068	17.0 ± 0.015
α-Globulins	11.7 ± 0.0094	19.9 ± 0.021
β-Globulins	7.8 ± 0.010	13.8 ± 0.027
γ-Globulins	13.7 ± 0.0082	10.4 ± 0.014
Casein	10.0 ± 0.011	22.0 ± 0.023
α-Lactalbumin	11.0 ± 0.0062	17.0 ± 0.0099
β-Lactoglobulin	15.0 ± 0.0043	20.1 ± 0.015
Immune Globulins	13.3 ± 0.018	10.2 ± 0.0048
"Blood" Serum Albumin	9.7 ± 0.039	16.7 ± 0.043

TABLE 5. THE RADIOACTIVITY OF URINE FROM X-35 (LACTATING RABBIT 13TH-17TH DAYS POSTPARTUM INJECTED WITH 45.0 UC. OF DL-LEUCINE-2-C^{14}).

hrs. post inj.	ml. urine counted	CPM[1]	CPM/ml.
0.16	0.5	642	1284
2	1.0	527	527
6	0.6	145	252
12	1.2	22	18
24	1.0	---	---
36	1.0	---	---
48	1.0	---	---
72	1.0	---	---

[1] The counts per minute were not significantly different from background after 12 hours post injection.

TABLE 6. SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-18, 10TH-13TH DAYS POST-PARTUM) INJECTED WITH 0.31 μC. (400 mg.) C^{14} LABELED γ-GLOBULINS

hrs. post. inj.	DPM	mg. protein counted	$\mu c./mg. \times 10^4$
		Blood Albumin	
2	1,146	44.2	0.166
6	978	45.0	0.098
12	965	47.8	0.091
24	792	44.9	0.076
48	647	38.9	0.075
72	460	30.5	0.068
		Blood α-Globulins	
2	822	40.6	0.091
6	696	36.7	0.085
12	618	32.4	0.086
24	639	39.8	0.072
48	555	41.5	0.060
72	448	35.3	0.057
		Blood β-Globulins	
2	735	40.0	0.083
6	954	45.5	0.095
12	816	42.3	0.087
24	864	46.9	0.085
48	630	36.7	0.077
72	620	39.8	0.070
		Casein From Milk	
2	1,122	45.2	0.112
6	1,062	49.0	0.098
12	935	45.7	0.092
24	939	50.4	0.084
48	841	47.3	0.080
72	811	47.8	0.077
		Milk β-Lactoglobulin	
2	648	49.9	0.058
6	384	21.8	0.079
12	646	45.5	0.064
24	600	45.9	0.059
48	462	40.6	0.051
72	515	38.7	0.060

TABLE 6. (CONT.) SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-18, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 0.31 μc. (400 mg.) C^{14} LABELED γ-GLOBULINS

hrs. post inj.	DPM	mg. protein counted	μc./mg. x 10^4
		Milk α-Lactalbumin	
2	720	45.9	0.071
6	811	45.7	0.080
12	776	49.1	0.071
24	669	47.5	0.064
48	558	46.5	0.054
72	544	47.4	0.052

TABLE 7. SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-19, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 2.0 μC. (800 Mg.) OF C^{14} LABELED γ-GLOBULINS

hrs. post inj.	DPM	mg. protein counted	μc./mg. x 10^4
		Blood Albumin	
2	978	39.5	0.112
6	762	50.2	0.069
12	1,008	45.0	0.101
24	815	41.3	0.089
48	972	48.6	0.090
72	921	50.0	0.083
		Blood α-Globulins	
2	670	29.3	0.103
6	1,369	40.0	0.154
12	1,152	44.8	0.116
24	855	38.5	0.100
48	835	42.2	0.089
72	648	35.6	0.082
		Blood β -Globulins	
2	342	26.0	0.059
6	696	47.6	0.073
12	378	26.1	0.065
24	477	35.2	0.061
48	520	40.8	0.058
72	430	38.9	0.050
		Casein From Milk	
2	1,074	40.9	0.118
6	1,410	48.7	0.130
12	912	42.0	0.098
24	911	45.5	0.090
48	896	47.8	0.085
72	744	44.4	0.076
		Milk β -Lactoglobulin	
2	398	9.7	0.185
6	254	5.3	0.216
12	253	6.0	0.190
24	334	8.4	0.179
48	284	7.5	0.171
72	320	8.8	0.164

TABLE 7. (CONT.) SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-19, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 2.0 μC. (800 mg.) OF C^{14} LABELED γ-GLOBULINS

hrs. post inj.	DPM	mg. protein counted	μc./mg. x 10^4
		Milk α-Lactalbumin	
2	954	46.1	0.093
6	636	46.5	0.062
12	945	40.1	0.106
24	696	44.3	0.071
48	635	42.0	0.068
72	635	45.8	0.063

TABLE 8. SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-17, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 5.16 μc. (2.0 gm.) C^{14} LABELED ALBUMIN.

hrs. post inj.	DPM	mg. protein counted	μc./mg. x 10^4
Casein From Milk			
2	1,170	41.4	0.127
6	1,130	46.3	0.110
12	1,091	45.5	0.108
24	960	40.9	0.106
48	905	44.3	0.092
72	935	49.0	0.086
Milk β -Lactoglobulin			
2	754	33.9	0.125
6	583	26.0	0.101
12	759	30.0	0.114
24	840	35.5	0.106
48	684	32.4	0.095
72	705	36.1	0.088
Milk α-Lactalbumin			
2	960	40.9	0.106
6	213	8.8	0.109
12	906	35.9	0.113
24	630	33.6	0.085
48	714	43.4	0.074
72	840	38.4	0.099
Milk Immune Globulins			
2	725	40.0	0.082
6	1,230	44.6	0.124
12	866	38.7	0.101
24	910	42.5	0.096
48	831	45.8	0.084
72	668	39.0	0.077
Blood γ -Globulins			
2	311	11.3	0.124
6	380	14.4	0.119
12	366	18.8	0.088
24	524	27.4	0.086
48	368	22.5	0.074
72	470	30.0	0.071

TABLE 8. (CONT.) SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-17, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 5.16 μC. (2.0 gm.) C^{14} LABELED ALBUMIN.

hrs. post inj.	DPM	mg. protein counted	μc./mg. x 10^4
		Blood α -Globulins	
2	465	27.4	0.077
6	546	25.7	0.096
12	701	35.7	0.089
24	535	31.2	0.077
48	410	26.0	0.071
72	549	38.5	0.064
		Blood β -Globulins	
2	643	26.1	0.112
6	840	31.4	0.141
12	780	35.5	0.099
24	816	40.6	0.088
48	591	32.3	0.083
72	670	41.0	0.074

TABLE 9. SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-20, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 13.2 μC. (3.0 gm.) OF C^{14} LABELED ALBUMIN.

hrs. post inj.	DPM	mg. protein counted	μc./mg. protein x 10^4
		Blood γ -Globulins	
2	1,152	41.4	0.126
6	1,020	35.1	0.131
12	1,219	45.6	0.119
24	885	39.8	0.101
48	870	40.0	0.098
72	1,014	49.7	0.092
		Blood α -Globulins	
2	1,074	22.1	0.219
6	930	38.1	0.109
12	784	32.5	0.110
24	626	29.6	0.095
48	711	36.4	0.088
72	785	42.9	0.082
		Blood β -Globulins	
2	1,296	23.8	0.245
6	1,170	46.4	0.114
12	830	39.0	0.096
24	582	35.6	0.075
48	666	42.3	0.071
72	626	48.7	0.058
		Casein From Milk	
2	762	46.0	0.0750
6	984	44.7	0.0990
12	866	48.1	0.0812
24	875	49.5	0.0796
48	641	49.9	0.0580
72	421	47.3	0.0401
		Milk Immune Globulins	
2	275	10.4	0.119
6	1,010	36.7	0.124
12	623	24.8	0.113
24	763	39.5	0.087
48	750	41.1	0.082
72	774	45.3	0.078

TABLE 9. (CONT.) SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-20, 10TH-13TH DAYS POSTPARTUM) INJECTED WITH 13.2 μC. (3.0 gm.) OF C^{14} LABELED ALBUMIN.

hrs. post inj.	DPM	mg. protein counted	μc./mg. protein x 10^4
		Milk β-Lactoglobulin	
2	786	30.2	0.117
6	328	11.0	0.134
12	724	29.9	0.109
24	755	35.8	0.095
48	541	30.5	0.080
72	710	39.0	0.082
		Milk α-Lactalbumin	
2	390	21.7	0.081
6	526	25.0	0.095
12	577	29.9	0.087
24	328	20.5	0.072
48	356	24.3	0.066
72	380	27.2	0.063

TABLE 10. SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-24, 10TH-11TH DAYS POSTPARTUM) INJECTED WITH 1.63 μC. (492 mg.) C^{14} LABELED α-GLOBULINS

hrs. post inj.	DPM	mg. protein counted	μc./mg. proteins x 10^5
		Blood α-Globulins	
0.16	107,100	25.6	188.000
2	79,150	20.2	177.000
6	107,250	30.3	160.000
12	65,950	20.3	146.000
24	35,400	13.1	123.000
		Blood Albumin	
2	609.0	51.6	0.531
6	500.0	46.3	0.487
12	954.5	51.3	0.838
24	543.0	49.4	0.496
		Blood γ-Globulins	
2	362.0	38.1	0.428
6	363.9	22.2	0.779
12	1,046.0	32.0	1.472
24	771.5	47.6	0.725
		Blood β-Globulins	
2	938.0	44.6	0.946
6	1,868.0	39.4	2.019
12	1,683.0	33.5	2.285
24	639.5	27.2	1.059
		Milk Immune Globulins	
2	328.0	50.5	0.293
6	510.2	48.4	0.477
12	1,478.5	50.3	1.324
24	687.0	51.6	0.599
		Casein	
2	1,122	52.2	0.969
6	1,416	53.2	1.199
12	1,008	51.1	0.884
24	714	51.0	0.631

TABLE 10. (CONT.) SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-24, 10TH-11TH DAYS POSTPARTUM) INJECTED WITH 1.63 µC. (492 mg.) C^{14} LABELED α-GLOBULINS

hrs. post inj.	DPM	mg. protein counted	µc./mg. proteins x 10^5
		Milk α-Lactalbumin	
2	354.0	36.5	0.437
6	260.5	25.1	0.514
12	297.5	21.7	0.617
24	400.0	9.2	1.950
		Milk β-Lactoglobulin	
2	601	49.6	0.564
6	906	32.2	1.270
12	816	46.0	0.802
24	449	48.3	0.419

TABLE 11. SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-27, 10TH-11TH DAYS POSTPARTUM) INJECTED WITH 2.37 μC. (398 mg.) OF C^{14} LABELED β -GLOBULINS

hrs. post. inj.	DPM	mg. protein counted	μc./mg. protein x 10^5
		Blood β -Globulins	
0.16	366,900	45.2	366.000
2	289,500	46.7	278.000
6	220,000	42.3	234.000
12	238,500	50.5	212.000
24	174,000	53.9	145.000
		Blood Albumins	
2	122.0	53.1	0.104
6	454.0	49.9	0.409
12	884.0	50.9	0.784
24	1,067.0	49.4	0.939
		Blood γ -Globulins	
2	424.0	40.0	0.478
6	716.0	41.0	0.788
12	1,309.0	54.5	1.080
24	434.0	24.7	0.794
		Blood α -Globulins	
2	665.5	32.8	0.915
6	1,271.0	28.6	2.010
12	918.0	40.3	1.030
24	548.0	10.0	2.460
		Casein	
2	2,934	48.5	2.720
6	4,338	53.7	3.640
12	5,478	51.4	3.930
24	4,626	50.7	3.580
		Milk Immune Globulins	
2	420.0	52.5	0.361
6	371.5	45.9	0.364
12	676.0	43.4	0.703
24	714.0	48.5	0.708

TABLE 11. (CONT.) SPECIFIC ACTIVITIES OF BLOOD AND MILK PROTEIN FRACTIONS FROM A LACTATING RABBIT (NO. X-27, 10TH-11TH DAYS POSTPARTUM) INJECTED WITH 2.37 µC. (398 mg.) OF C^{14} LABELED β-GLOBULINS

hrs. post inj.	DPM	mg. protein counted	µc./mg. protein x 10^5
		α-Lactalbumin	
2	276.1	31.4	0.396
6	199.0	15.2	0.590
12	242.0	14.4	0.758
24	440.0	20.0	0.991
		β-Lactoglobulin	
2	1,008	34.8	1.310
6	1,062	17.7	3.520
12	1,704	33.1	2.320
24	1,188	37.7	1.420

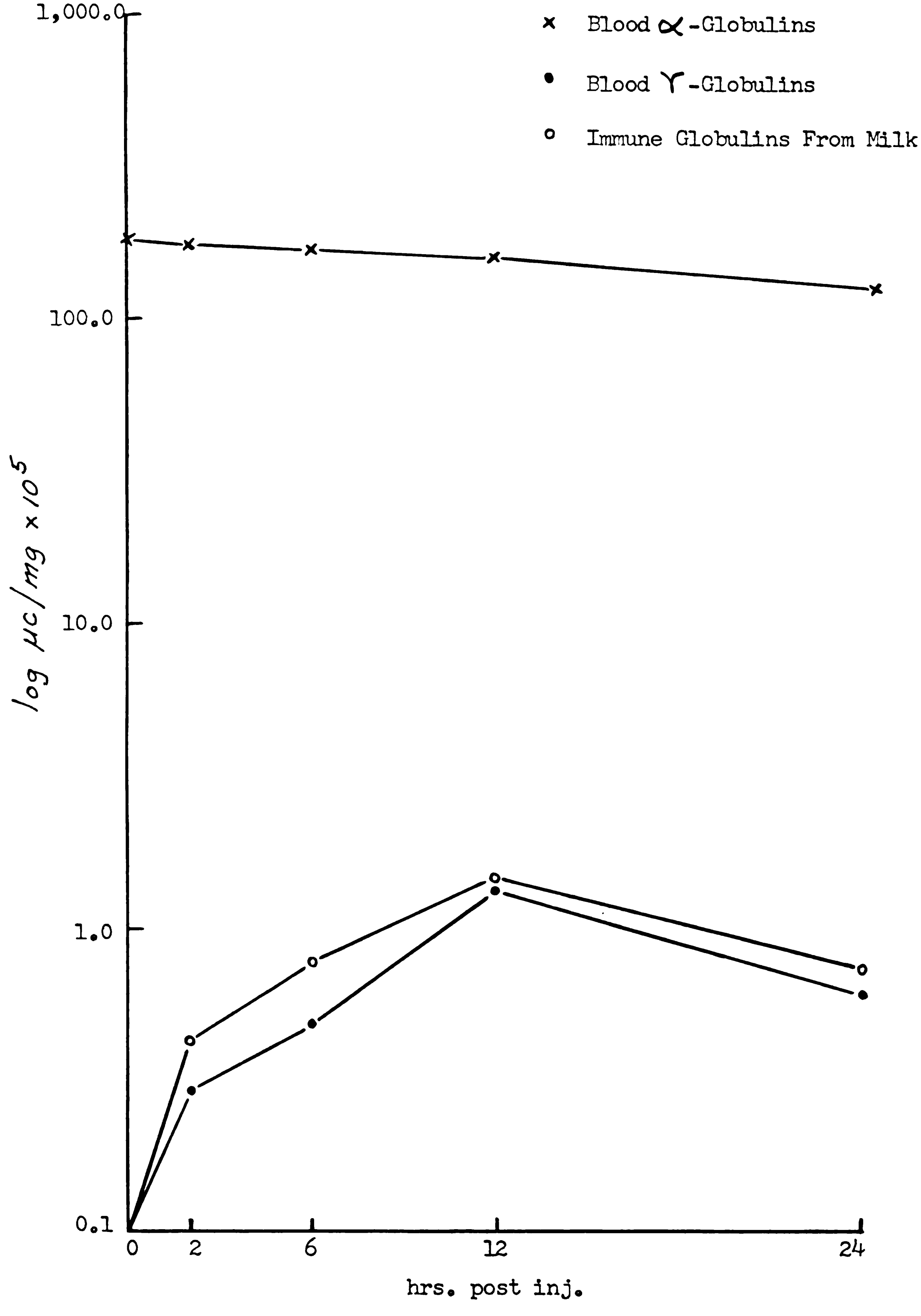

FIGURE 4. THE LOG OF SPECIFIC ACTIVITY (μc./mg. x 10^5) VS. TIME (hrs. post inj.) FOR BLOOD α-GLOBULINS, BLOOD γ-GLOBULINS AND IMMUNE GLOBULINS FROM MILK ISOLATED FROM X-24 (LACTATING RABBIT 10TH-11TH DAYS POSTPARTUM INJECTED WITH 1.63 μC. OF C^{14} LABELED α-GLOBULINS).

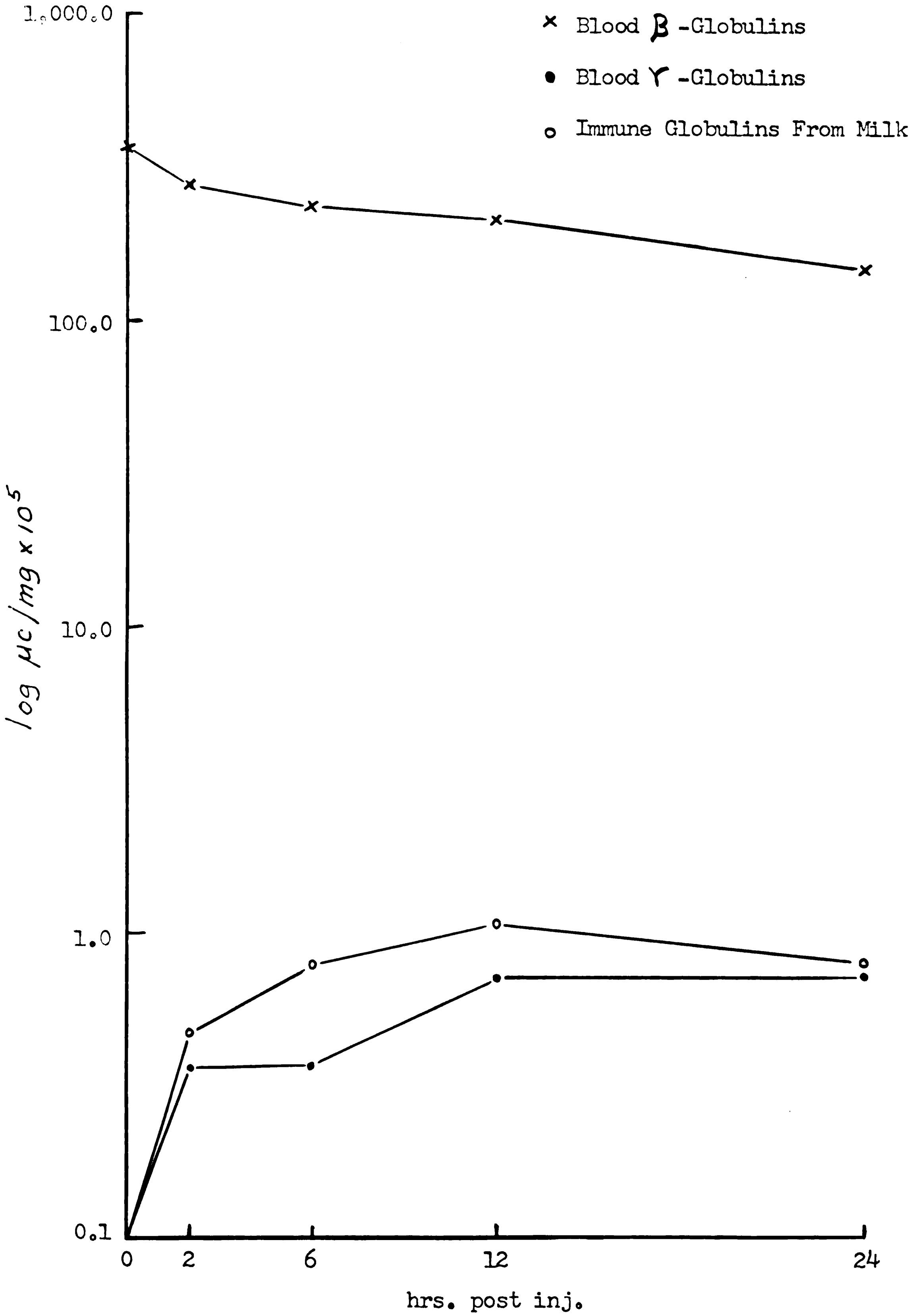

FIGURE 5. THE LOG OF SPECIFIC ACTIVITY (μc./mg. x 10^5) VS. TIME (hrs. post inj.) FOR BLOOD β-GLOBULINS, BLOOD γ-GLOBULINS AND IMMUNE GLOBULINS FROM MILK ISOLATED FROM X-27 (LACTATING RABBIT 10TH-11TH DAYS POSTPARTUM INJECTED WITH 2.37 μC. OF C^{14} LABELED β-GLOBULINS).