Smith, Diane H.

INSULIN RECEPTORS IN THE MAMMARY GLAND

The Ohio State University PH.D. 1986

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __✓__.

1. Glossy photographs or pages _____
2. Colored illustrations, paper or print _____
3. Photographs with dark background _____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages __✓__
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Dissertation contains pages with print at a slant, filmed as received _____
16. Other _____

_____

_____

University
Microfilms
International

INSULIN RECEPTORS IN THE MAMMARY GLAND

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

Diane H. Smith, B.S., M.S.

* * * * *

The Ohio State University

1986

Dissertation Committee:

D. L. Palmquist

F. L. Schanbacher

K. L. Roehrig

Approved By

Adviser

Department of Dairy Science

PREFACE

I can do all things through Christ Who strengthens me.

Philippians 4:13

DEDICATION

To my Heavenly Father, apart from Whom I am nothing.

ACKNOWLEDGEMENTS

I would like to thank my adviser, Dr. Donald L. Palmquist, and the members of my reading committee, Drs. K. L. Roehrig and F. L. Schanbacher, for their constructive criticisms of this dissertation and for their encouragement during the course of these studies.

I am extremely grateful to Drs. T. D. Etherton and C. R. Baumrucker of The Pennsylvania State University for teaching me the insulin receptor methodology.

I am also indebted to Dr. A. E. Staubus for helping me analyze the insulin binding data.

A very special thanks is due Dr. H. R. Conrad, whose belief in me was a constant source of strength to me during this research.

Thanks also go to Barb Gerard for generously giving of her time and herself in the typing of this dissertation.

I would like to acknowledge two very special people, Bill Weiss and Ting Ting Liu, for their help with my

statistical analyses and graphics, but most of all, for helping me to see the funny side of every situation.

The friendship and assistance of Judy Riggenbaugh, Karen Scheibe, and Michele Martino will always be treasured.

I am extremely grateful to the members of Orrville Community Church for their fellowship, prayers, and genuine concern for me. You'll never know how important you were!

To some very special friends along the way - Brenda Armstrong, Rosalie Green, Karen and Gary Coffing, Mary and Joe Gnizack, Hazel Zun, Connie Miller, Pam Maxwell, Pat Tirabasso, Barb Thuma and Paula Lundin. You were the people that made it all worthwhile.

Sincere thanks are extended to my mother, my father, and my Aunt BZ, who never gave up on me. Guess what? I finally made it! Thanks so much for your love and support. They were indispensable.

To my husband and best friend, Don, (alias Gourd Head) who kept me laughing during the past seven years. I love you for bringing such joy to my life.

Finally, I want to thank my Lord and Savior, Jesus Christ, for teaching me the most valuable lesson of all, how to be content in all circumstances.

VITA

April 16, 1955	Born - Sayre, Pennsylvania
May, 1977.	B.S., The Pennsylvania State University, University Park, Pennsylvania
December, 1979	M.S., Virginia Polytechnic Institute and State University, Blacksburg, Virginia
January, 1980-August, 1983 . . .	Graduate Research Associate, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio
September, 1983-August, 1984 . .	Presidential Fellow, The Ohio State University
August, 1984-Present	Graduate Student, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio

PUBLICATIONS

Smith, D. H., D. L. Palmquist, and F. L. Schanbacher. 1986. Characterization of insulin binding to bovine liver and mammary microsomes. Comp. Biochem. Physiol. (in press).

Gleockler, D. H., F. L. Schanbacher, and D. L. Palmquist. 1984. Insulin binding in bovine mammary membranes. Fed. Proc. 43:524 (abstr.)

Gleockler, D. H., D. L. Palmquist, and T. D. Etherton. 1983. Characterization of insulin binding in bovine liver microsomes. J. Dairy Sci. 66(Suppl. 1):234 (abstr.).

Gleockler, D. H., T. D. Etherton, and C. R. Baumrucker. 1983. Insulin receptors of bovine lactating mammary tissue. The 1983 Pennsylvania Dairy and Livestock Day Proceedings.

Gleockler, D. H., L. F. Ferreri, and E. Flaim. 1980. Lipoprotein patterns in normal and lactating Holstein cows bled at various times: Effects of milking. Proc. Soc. Exper. Biol. Med. 165:118.

Gleockler, D. H. 1979. Lipoprotein studies in the dairy cow. M.S. thesis. The Virginia Polytechnic Institute and State University.

Ferreri, L. F. and D. H. Gleockler. 1979. Electrophoretic characterization of bovine lipoprotein subfractions isolated by agarose gel chromatography. J. Dairy Sci. 62:1577.

Gleockler, D. H. and L. F. Ferreri. 1979. Effect of bleeding time on bovine lipoprotein concentrations. J. Dairy Sci. 62(Suppl. 1):191.

Ferreri, L. F. and D. H. Gleockler. 1979. Further characterization of bovine high density lipoproteins. J. Dairy Sci. 62(Suppl. 1):190 (abstr.).

Ferreri, L. F. and D. H. Gleockler. 1979. Heterogeneity of bovine serum lipoproteins. J. Dairy Sci. 62(Suppl. 1):215 (abstr.).

Gleockler, D. H. and L. F. Ferreri. 1979. Screening dairy cows for variations in their lipoprotein profiles. J. Dairy Sci. 62(Suppl. 1):197 (abstr.).

FIELDS OF STUDY

Major Field: Nutritional Biochemistry

Minor Field: Endocrinology

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The role that insulin plays in regulating nonruminant mammary metabolism is not well established. Mammary tissue requires insulin for survival in vitro (11). Furthermore, insulin, in combination with prolactin and adrenal corticoid is necessary for maximal stimulation of milk lipid (5, 32) and protein (34) synthesis. On the other hand, insulin is not essential to lactose synthesis (4) nor glucose uptake (24) by the mammary gland. Therefore, insulin appears to act in a permissive rather than a regulatory capacity with respect to most mammary cell functions.

Recently, Jones et al. (21) reported that infusion of insulin at a dosage which led to a small physiological rise in plasma insulin concentration without causing marked hypoglycemia stimulated lipogenic rates in rat mammary gland by 100%. They concluded that the lactating mammary gland is a highly insulin-sensitive tissue. In addition, Threadgold and Kuhn (35) demonstrated that transport of 3-0-methylglucose by the mammary glands of

starved rats can be restored toward normal by insulin. These two studies indicate that insulin may be more important in regulating mammary metabolism than the majority of evidence implies.

An in vitro insulin requirement for mammary secretory cell maintenance (11) suggests that insulin works directly on the mammary cell. Moreover, the fact that only small changes in insulin concentration are required to produce large rate changes in mammary lipogenesis (21) implies that plasma insulin concentration by itself is not responsible for the high sensitivity of the gland to insulin. Insulin receptors have been identified in murine mammary cells (9, 17, 18, 27). Furthermore, insulin binding to murine mammary cells is greater during lactation than during pregnancy (9, 18, 27). Physiological regulation of insulin binding to the mammary gland suggests that the responsiveness of the gland can be altered, at least in part, via the insulin receptor.

Since glucose metabolism is known to differ between ruminants and nonruminants (22), the role that insulin plays in regulating ruminant mammary metabolism is particularly unclear. Little glucose is absorbed from the gut of the ruminant (22); hence, endogenous glucose production must meet the animal's glucose needs.

Gluconeogenesis is permanently active in the ruminant (22), occurring primarily in the liver (3). As a result, blood glucose and insulin concentrations are lower in ruminants (14) than in nonruminants (38). In addition, ruminants seem less sensitive to insulin than nonruminants. Blood glucose concentrations in diabetic sheep respond slowly to insulin administration (4 d) (20), but the same response in the nonruminant occurs rapidly (30 min) (12).

As in the nonruminant, insulin is required for viability of ruminant mammary tissue in vitro (6) and maintenance of secretory activity in vivo (26). Insulin also does not appear to be required for lactose synthesis (16) and glucose transport (15) in the mammary gland of the ruminant. Conversely, ruminant mammary tissue seems less responsive to insulin than nonruminant mammary tissue with respect to lipogenesis since insulin added in vitro to ruminant mammary tissue in the presence of acetate and glucose has no stimulatory effect (2).

Vernon et al. (36) postulated that removal of adipose tissue from the cow results in temporary refractoriness to insulin stimulation. These workers found that bovine adipocytes incubated for only 24 hr in vitro responded only slightly to insulin stimulation of glucose uptake and

pyruvate kinase activity. On the other hand, adipocytes incubated for 48 hr in vitro responded greatly to insulin stimulation. Temporary refractoriness of bovine mammary tissue in culture could account for the inability of previous investigators (2) to detect a stimulatory effect of insulin on lipogenesis.

Although insulin is essential for normal mammary function both in vitro (6) and in vivo (26) in the ruminant, the insulin-insensitivity of the gland seems to rule out a regulatory role for insulin. Nonetheless, nonruminant mammary tissue also appears refractory to insulin stimulation with respect to lactose synthesis (4) and glucose uptake (24); yet, certain physiological conditions, such as lactation, result in upregulation of insulin receptor numbers on the mammary cell (9, 18) implicating insulin in the regulation of mammary metabolism. With this in mind, examination of insulin receptor regulation in the mammary gland of the ruminant was indicated.

At the time the experiments in this dissertation were initiated, insulin receptors had not been identified in bovine mammary tissue. Insulin receptors had been identified, however, in bovine liver microsomes (30). Since a

thorough characterization of insulin receptors is essential to future studies dealing with insulin receptor regulation in the mammary gland of the ruminant, insulin receptors in bovine mammary microsomes were characterized. Insulin receptors in bovine liver microsomes were also studied for comparative purposes, as well as for technique verification.

Posner et al. (29) reported significant tissue and species differences in insulin binding. Such differences could indicate differential insulin requirements, both among species and among tissues within species. In view of the differences between ruminants and nonruminants in glucose metabolism and insulin regulation thereof (20), differences in insulin binding between these two animal groups might be expected. Therefore, insulin binding by liver and mammary microsomes of the pig and dairy cow, two species not examined by Posner et al. (29), was examined and compared.

In the aforementioned studies, a microsomal model was chosen because microsomal preparation is economical and convenient in that large quantities of tissue can be prepared at one time and frozen for future use. Unfortunately, insulin binding to the microsomal fraction is not

necessarily representative of insulin binding to the plasma membrane, as microsomes are composed of rough endoplasmic reticulum, smooth endoplasmic reticulum, and Golgi membranes as well (8). Milk, however, contains an abundance of membranes derived from the plasma membrane of the mammary secretory cell.

When milk fat is secreted into the alveolar lumen, the fat droplet is coated with a portion of the apical plasma membrane of the mammary secretory cell (25). These milk fat globule membranes, MFGM, are easily isolated from milk in large quantities (7) and contain numerous proteins (25, 37). Flint and West (10) reported the presence of insulin receptors in MFGM isolated from murine and caprine milk.

Since MFGM contain insulin receptors and are thought to originate from the plasma membrane of the mammary secretory cell, they may provide a more accurate model than microsomes for assessing insulin binding to the mammary gland. Furthermore, the model is advantageous in that repeat sampling of animals is possible. For this reason, insulin binding to bovine MFGM was assessed and compared to binding by bovine mammary microsomes. In addition, the association between insulin binding to MFGM and physiological and nutritional status was examined to

determine if MFGM could be used to monitor insulin receptor changes in the mammary gland.

High fat diets have been shown to decrease insulin binding by rat adipocytes (19) and liver plasma membranes (33), primarily be decreasing the number of insulin receptors available for binding. High fat diets have also been associated with insulin resistance in the ruminant. Dairy cattle fed a high fat diet exhibit impaired glucose clearance and enhanced insulin secretion in response to a glucose challenge (28). Interestingly, milk protein yield (1, 23, 31) and percentage (23, 31) are depressed in dairy cattle fed a high fat diet. Based on the stimulatory role of insulin in protein synthesis (13), it is possible that dietary fat inhibits milk protein synthesis by modulating mammary insulin receptors. Therefore, the effect of fat feeding on insulin binding to rat mammary and liver microsomes was examined. Although the primary emphasis of this dissertation was insulin binding by bovine mammary membranes, rats were employed in this study for economical and practical reasons. Liver, once again, was included for comparative purposes.

In summary, the objectives of this dissertation were as follows:

1. To characterize insulin binding to bovine mammary microsomes and to determine if the specificity and kinetics of this binding indicate the presence of insulin receptors in bovine mammary gland;

2. To examine and compare insulin binding by liver and mammary microsomes of the pig and dairy cow;

3. To examine insulin binding to bovine MFGM and to evaluate this model's usefulness in assessing insulin receptor regulation in the mammary gland; and

4. To examine the effect of dietary fat on insulin binding by rat mammary and liver microsomes.

LIST OF REFERENCES

1. Banks, W., J. L. Clapperton, M. E. Ferrie, and A. G. Wilson. 1976. Effect of feeding fat to dairy cows receiving a fat-deficient basal diet. J. Dairy Res. 43:213.

2. Bauman, D. E., D. L. Ingle, R. W. Mellenberger, and C. L. Davis. 1973. Factors affecting in vitro lipogenesis by bovine mammary tissue slices. J. Dairy Sci. 56:1520.

3. Bergman, E. M., R. P. Brockman, and C. F. Kaufman. 1974. Glucose metabolism in ruminants: Comparison of whole-body turnover with production by gut, liver,and kidneys. Fed. Proc. 33:1849.

4. Carrick, D. T. and N. J. Kuhn. 1978. Diurnal variation and response to food withdrawal of lactose synthesis in lactating rats. 1978. Biochem. J. 174:319.

5. Carrington, C. A., H. L. Hosick, I. A. Forsyth, and R. Dils. 1983. Milk-fat synthesis by globules prepared from rabbit mammary gland: Response to insulin, corticosterone, prolactin and progesterone. J. Endocrin. 97:157.

6. Collier, R. J., D. E. Bauman, and R. L. Hays. 1977. Lactogenesis in explant culture of mammary tissue from pregnant cows. Endocrinology 100:1192.

7. Dowben, R. M., J. R. Brunner, and D. E. Philpott. 1967. Studies on milk fat globule membranes. Biochim. Biophys. Acta 135:1.

8. Ehrenreich, J. R., J. J. M. Bergeron, C. P. Siekevitz, and G. E. Palade. 1978. Golgi fractions prepared from rat liver homogenates. I. Isolation procedure and morphological characterization. J. Cell Biol. 59:45.

9. Flint, D. J. 1982. Insulin binding to rat mammary gland at various stages of cell isolation and purification. Mol. Cell. Endocrinol. 26:281.

10. Flint, D. J. and D. W. West. 1983. Insulin receptors in milk. Br. Vet. J. 139:39.

11. Forsyth, I. A. 1983. The endocrinology of lactation. Pages 309-349 in Biochemistry of lactation. T. B. Mepham, ed. Elsevier Science Publishers, BV, New York, NY.

12. Ganong, W. F. 1975. Page 253 in Review of medical physiology. 7th ed. Lange Medical Publications, Los Altos, CA.

13. Guyton, A. C. 1976. Page 1042 in Textbook of medical physiology. W. B. Saunders Co., Philadelphia, PA.

14. Horino, M., L. J. Machlin, F. Hertelendy, and D. M. Kipnis. 1968. Effect of short-chain fatty acids on plasma insulin in ruminant and nonruminant species. Endocrinology 83:118.

15. Hove, K. 1978. Effects of hyperinsulinemia on lactose secretion and glucose uptake by the goat mammary gland. Acta Physiol. Scand. 104:422.

16. Hove, K. 1978. Maintenance of lactose secretion during acute insulin deficiency in lactating goats. Acta Physiol. Scand. 103:173.

17. Inagaki, Y. and K. Kohmoto. 1981. A method for the iodination of insulin and its binding to dissociated mouse mammary cells. Endocrinol. Japon. 28:29.

18. Inagaki, Y. and K. Kohmoto. 1982. Changes in Scatchard plots for insulin binding to mammary epithelial cells from cycling, pregnant, and lactating mice. Endocrinology 110:176.

19. Ip, C., H. M. Tepperman, P. Holohan, and J. Tepperman. 1976. Insulin binding and insulin response of adipocytes from rats adapted to fat feeding. J. Lipid Res. 17:588.

20. Jarrett, I. G., O. H. Filsell, and F. J. Ballard. 1974. Metabolic and endocrine interrelationships in

normal and diabetic sheep. Horm. Metab. Res. Suppl. Series 4:111.

21. Jones, R. G., V. Ilic, and D. H. Williamson. 1984. Physiological significance of altered insulin metabolism in the conscious rat during lactation. Biochem. J. 220:455.

22. Lindsay, D. B. 1978. Gluconeogenesis in ruminants. Biochem. Soc. Trans. 6:1152.

23. MacLeod, G. K., Y. Yu, and L. R. Schaeffer. 1977. Feeding value of protected animal tallow for high yielding dairy cows. J. Dairy Sci. 60:726.

24. Martin, R. J. and R. L. Baldwin. 1971. Effects of insulin and anti-insulin serum treatments on levels of metabolites in rat mammary glands. Endocrinology 88:868.

25. Mather, I. H. and T. W. Keenan. 1983. Function of endomembranes and the cell surface in the secretion of organic milk constituents. Pages 232-283 in Biochemistry of lactation. T. B. Mepham, ed. Elsevier Science Publishers, BV, New York, NY.

26. Nowak, J. and L. Dzialoszynski. 1967. Effect of experimental alloxan diabetes on the secretion and composition of goat milk. Acta Physiol. Pol. 18:595.

27. O'Keefe, E. and P. Cuatrecasas. 1974. Insulin receptors in murine mammary cells: Comparison in pregnant and nonpregnant animals. Biochim. Biophys. Acta 343:64.

28. Palmquist, D. L. and E. A. Moser. 1981. Dietary fat effects on blood insulin, glucose utilization, and milk protein content of lactating cows. J. Dairy Sci. 64:1664.

29. Posner, B. I., P. A. Kelly, R. P. C. Shiu, and H. G. Friesen. 1974. Studies of insulin, growth hormone and prolactin binding: Tissue distribution, species variation and characterization. Endocrinology 95:521.

30. Rosen, P., B. Ehrich, E. Junger, H. J. Bubenzer, and L. Kuhn. 1979. Binding and degradation of insulin by plasma membranes from bovine liver isolated by

large scale preparation. Biochim. Biophys. Acta 587:593.

31. Storry, J. E., P. E. Brumby, A. J. Hall, and V. W. Johnson. 1974. Response of the lactating cow to different methods of incorporating casein and coconut oil in the diet. J. Dairy Sci. 57:61.

32. Strong, C. R., I. Forsyth, and R. Dils. 1972. The effects of hormones on milk-fat synthesis in mammary explants from pseudopregnant rabbits. Biochem. J. 128:509.

33. Sun, J. V., H. M. Tepperman, and J. Tepperman. 1977. A comparison of insulin binding by liver plasma membranes of rats fed a high glucose diet or a high fat diet. J. Lipid Res. 18:533.

34. Terry, P. M., M. R. Banerjee, and R. M. Lui. 1977. Hormone-inducible casein messenger RNA in a serum-free organ culture of whole mammary gland. Proc. Natl. Acad. Sci. USA 74:2441.

35. Threadgold, L. C. and N. J. Kuhn. 1984. Monosaccharide transport in the mammary gland of the intact lactating rat. Biochem. J. 218:213.

36. Vernon, R. G., E. Finley, E. Taylor, and D. J. Flint. 1985. Insulin binding and action on bovine adipocytes. Endocrinology 116:1195.

37. Wiman, K., B. Curman, L. Tragardh, and P. A. Peterson. 1979. Demonstration of HLA-DR-like antigens on milk fat globule membranes. Eur. J. Immunol. 9:190.

38. Yalow, R. S. and S. A. Berson. 1960. Immunoassay of endogenous plasma insulin in man. J. Clin. Invest. 39:1157.

CHAPTER II

Characterization of Insulin Binding to Bovine Liver and Mammary Microsomes

INTRODUCTION

The role of insulin in metabolism of the mammary gland is uncertain, particularly in the ruminant where low blood glucose (Bergman et al, 1970) and low insulin concentrations (Trenkle, 1972) prevail. Studies with lactating rats (Martin and Baldwin, 1971) and goats (Hove, 1978) indicate that insulin is not required for glucose uptake by the mammary gland. Insulin is required, however, for maintenance of lactation, even in ruminants, since all secretory activity in alloxan-diabetic goats is lost irreversibly after several weeks (Nowak and Dzialoszynski, 1967). Insulin is essential to the formation of alpha-lactalbumin, casein (Nicholas and Topper, 1983), and casein mRNA (Bolander et al, 1981; Kulski et al, 1983) in rodent mammary tissues. Since these compounds are markers for the terminal differentiation of the mammary gland (Rutter et al, 1973;

Topper and Freeman, 1980), insulin appears necessary for overt maturation of the mammary epithelial cell.

Insulin interacts with a specific insulin receptor in the plasma membrane of the cell prior to eliciting its intracellular actions (Kahn, 1975); thus, both the receptor and insulin concentrations contribute to insulin action. Under certain physiological circumstances, insulin receptors are regulated by changes in receptor numbers and/or binding affinity (Olefsky and Ciaraldi, 1981), both of which alter the effective receptor concentration of the cell. Therefore, the stimulatory effect of insulin is a function of the effective receptor concentration and the plasma insulin concentration.

Receptor regulation may be of particular significance in ruminant mammary function. Lower insulin concentrations in plasma of ruminants would be inconsequential if receptor concentration is the definitive factor in cellular response. Insulin receptors have been characterized in several ruminant tissues (Grizard, 1983; Peacock et al, 1982; Haskell et al, 1984), including liver (Rosen et al, 1979; Gill and Hart, 1980), and adipose tissue (Vernon et al, 1985), but have not been identified in ruminant mammary gland. However, insulin receptors which are subject to regulation have been identified in murine mammary cells (Inagaki and Kohmoto, 1982). The objectives of this study

were: 1) to examine insulin binding to bovine liver and mammary microsomes; and 2) to determine if the specificity and kinetics of insulin binding indicate the presence of insulin receptors in bovine mammary tissue.

MATERIALS AND METHODS

Materials. Bovine crystalline insulin (26.6 U/mg), proinsulin, and glucagon were gifts of Eli Lilly Co. (courtesy of W. Fields). Carrier-free $Na^{125}I$ in 0.1M NaOH (high concentration) was purchased from New England Nuclear. Bovine serum albumin (BSA; fraction V) and guinea pig anti-bovine insulin serum (GPAIS) were obtained from Miles Biochemical. Phospholipase A_2 (PL-A_2) from bee venom (800 U/mg), phospholipase C (PL-C) (type 1) from Clostridium perfringens (20 U/mg), and bacitracin (50,000 U/g) came from Sigma Chemical Co.

Animals. Liver was obtained from three nonlactating Holstein cows (Bos taurus), 5 yr of age, and from three lactating cows (Bos taurus) (2 Holstein and 1 Jersey), 3 yr of age. Mammary tissue was obtained from six lactating Holstein cows, 3 - 9 yr of age. All animals were killed by a captive-bolt humane killer and the tissues removed as

soon as possible (average of 30 min postmortem). Upon removal, tissues were packed and held in ice until processing.

Preparation of Subcellular Fractions. Liver and mammary microsomes were prepared according to a modification of Pocius et al (1984). All steps were performed at 4°C. Tissues were minced finely in a buffer of 25 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl), 1 mM (ethylenedinitrilo)-tetraacetic acid (EDTA), 1 mM glutathione (reduced), and 250 mM sucrose, pH 7.5 at 4°C. Minced tissues were homogenized (1 g tissue to 4 ml buffer) using a Brinkman polytron, setting 7, with a PT-20ST generator, two homogenizations per mince, approximately 20 sec each. Since bovine mammary tissue contains substantial amounts of connective tissue, some mammary minces required a third homogenization for adequate liquification of the tissue. Homogenates were filtered sequentially through one, two, four, and eight layers of cheesecloth and then centrifuged at 9150 x g for 15 min. The resultant pellet (mitochondria, nuclei, and lysosomes) was discarded and the supernatant filtered through eight layers of cheesecloth. The filtrate was centrifuged again at 9150 x g for 15 min. The resultant supernatant was centrifuged at 100,000 x g for 1 hr. Microsomes (pellet) were resuspended in 50 mM

Tris-HCl, 10 mM $CaCl_2$ buffer, pH 7.5, at 4°C and stored at -20°C.

Membrane protein was usually determined using the Schacterle and Pollack modification (1973) of the Lowry protein assay (Lowry et al, 1951). However, in some cases, the method of Bradford (1976), was used. BSA was a standard for both procedures. Specific ^{125}I-insulin binding was directly proportional to microsomal protein concentration (data not shown). The protein concentration in all binding studies was 500 µg Lowry protein/ml unless otherwise specified.

Microsomes were assayed for insulin binding within 5 mon of storage.

Iodination of Insulin. Insulin was iodinated to a specific activity of 80 - 150 µCi/µg using chloramine T (Freychet et al, 1971). Ten µg (1 mg/ml) of bovine crystalline insulin were reacted with 2mCi $Na^{125}I$ (350-600 mCi/ml) and 10 µg chloramine T (1 mg/ml) for 15 - 20 sec. The reaction was terminated by diluting the mixture with 400 µl of 0.3 M sodium phosphate buffer containing 2.5% BSA, pH 7.4. The diluted mixture was transferred immediately to a 0.64 x 6.40 cm cellulose column (Whatman Cellulose Powder, CF11) and eluted with phosphate buffer without and with 12% BSA added to separate free ^{125}I from

protein-bound ^{125}I. All ^{125}I eluting with the 12% BSA buffer was considered to be protein bound and is referred to as the crude ^{125}I-insulin preparation. Prior to use in a radioreceptor assay, this crude preparation was purified on a Sephadex G-50 fine column (1.0 x 25.0 cm) eluted with 50 mM Tris-HCl buffer, pH 7.5. Integrity of the purified, labeled insulin was determined by precipitation with 10% trichloracetic acid (TCA) and GPAIS immunoprecipitation. Only ^{125}I-insulin which was at least 95% TCA precipitable and 93% immunoprecipitable was employed in these studies.

Standard Binding Assay for Liver Microsomes. Liver microsomes (500µg Lowry protein) were incubated with 0.1 pmole (0.6ng) ^{125}I-insulin in the absence or presence of 8.3 nmole (50µg) unlabeled insulin in 50 mM Tris-HCl, 0.1% BSA, pH 7.8 at 4°C. Incubations (1 ml final volume) were in polystyrene tubes (11 x 75 mm) at 4°C, in triplicate, with intermittent handmixing (ca every 8 hr). After 48 hr, the binding reaction was terminated by addition of 2 ml iced Tris-BSA buffer to each tube. Separation of bound vs free radioactivity was accomplished by a final centrifugation (4,080 x g) for 30 min at 4°C. Supernatant was decanted, the tubes were drained, and the inside walls above the pellet blotted with absorbent paper. Radioactivity in membrane pellets was counted for 10 min in

a gamma counter (The Nuclear Chicago 1185 Series, Automatic Gamma Counting System) at an average efficiency of 75%. Specific ^{125}I-insulin binding was determined as the difference between radioactivity bound in the absence (total binding) and the presence (nonspecific binding) of excess (50 µg/ml) unlabeled insulin. This amount was expressed routinely as a percentage of the total radioactivity added to each tube. Nonspecific binding by membranes averaged approximately 0.5% of the total radioactivity per incubation after accounting for radioactivity bound nonspecifically to the tube.

Standard Binding Assay for Mammary Microsomes. The standard mammary binding assay was similar to the standard liver binding assay with the following exceptions: Incubation buffer consisted of 50 mM Tris-HCl, 0.1% BSA, 1 mM bacitracin, pH 8.0 at 20°C. Incubations were at 20°C for 4.5 hr in polypropylene tubes (12 x 75 mm). Reactions were terminated by centrifugation at 19,600 x g for 30 min.

Insulin Degradation. Insulin integrity as a function of incubation time was evaluated at 20°C and 4°C in both liver and mammary microsomes. After the terminating centrifugation, the supernatants (1 ml) were precipitated

by addition of iced 10% TCA (1 ml). The TCA-insoluble fraction was pelleted by centrifugation at 1465 x g for 10 min at 4°C. Radioactivity in the pellets and corresponding supernatants was measured in the gamma counter. Intact radiolabeled protein was expressed as the percent TCA-precipitable radiolabeled material present.

Membrane Perturbation Studies. Cuatrecasas (1971a) and Cuatrecasas et al (1971) demonstrated increased insulin binding to adipose and liver plasma membranes, respectively, of rats by pretreating the membranes with various agents known to disrupt membrane phospholipid structure. They proposed that some of the receptors were normally buried in the membrane (cryptic), but that upon membrane perturbation, these sites were exposed and binding was thereby enhanced.

In this study, the presence of cryptic insulin receptors in bovine mammary microsomes was tested using PL-A_2, PL-C, and NaCl as membrane perturbents. Phospholipases catalyze the hydrolysis of membrane phospholipids; PL-A_2 specifically hydrolyzes the sn-2 ester linkage of the phospholipid, while PL-C splits the ester bond in sn-3, removing the phosphoryl base. The overall effect of both enzymes is to change the organization of membrane components. Likewise, addition of NaCl in high concentrations to the

incubation medium has been shown to perturb membrane organization (Cuatrecasas, 1971a).

The protocol used for the membrane perturbation experiment was that of Cuatrecasas et al (1971). Rat liver microsomes were also employed for comparison as well as for technique verification. Microsomes were prepared from livers of male Sprague Dawley rats as described above. Rat liver microsomes and bovine mammary microsomes (500 µg Lowry protein/ml) were incubated for 40 min at 37°C in Krebs Ringer Bicarbonate (KRB) buffer with 1% BSA in the absence (37° C Control) and presence of PL-C (50 µg/ml) or PL-A_2 (20 µg/ml). Microsomes were pelleted by centrifugation at 100,000 x g for 1 hr at 4°C and resuspended in KRB, 1% BSA at room temperature. Microsomes not incubated previously at 37°C also were suspended in KRB, 1% BSA with or without (20° C Control) 2 M NaCl at room temperature. All microsomal treatments were assayed for total insulin binding in the presence of 0.66 ng/ml ^{125}I-insulin for 40 min at 20°C. Nonspecific insulin binding was determined in parallel incubations containing 50 µg/ml unlabeled insulin. All incubations were performed in quintuplicate and terminated as in the standard binding assay for bovine mammary microsomes.

Analysis of Receptor Concentration and Binding Affinity. The binding parameters (binding affinities and receptor numbers) for insulin in bovine liver and mammary microsomes were determined by Scatchard analysis (1949). The Scatchard equation was fitted using the NONLIN program of Metzler et al (1974) for two classes of binding sites.

RESULTS AND DISCUSSION

Specific ^{125}I-insulin binding to both liver and mammary microsomes was time and temperature dependent (Figures 1 and 2, respectively). Equilibrium binding to liver microsomes was established by 48 hr at 4°C and remained stable for 72 hr (Figure 1). Equilibrium binding at 20°C was attained by 3 hr and was maintained until 16 hr; thereafter, binding decreased steadily to a minimum at 72 hr.

Freychet et al (1972) found that ^{125}I-insulin degradation by rat liver plasma membranes was significantly greater when incubated at 30°C than at 1°C. Therefore, the decrease in insulin binding post-16 hr at 20° C may be the result of increased insulin degradation at the higher incubation temperature. This is supported by the observation that decreased TCA precipitability of the supernatant

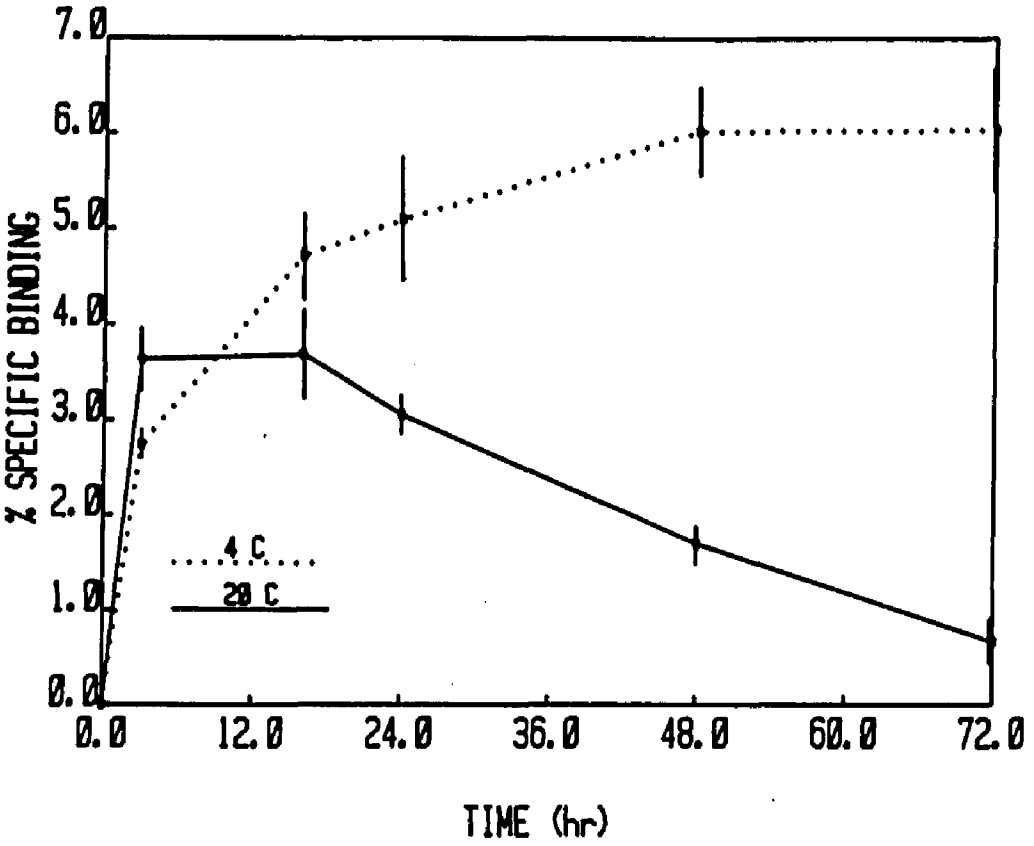

Figure 1. Time course of specific ^{125}I-insulin binding to bovine liver microsomes. Each point represents the mean of triplicate determinations for six animals. Bars represent the SEM.

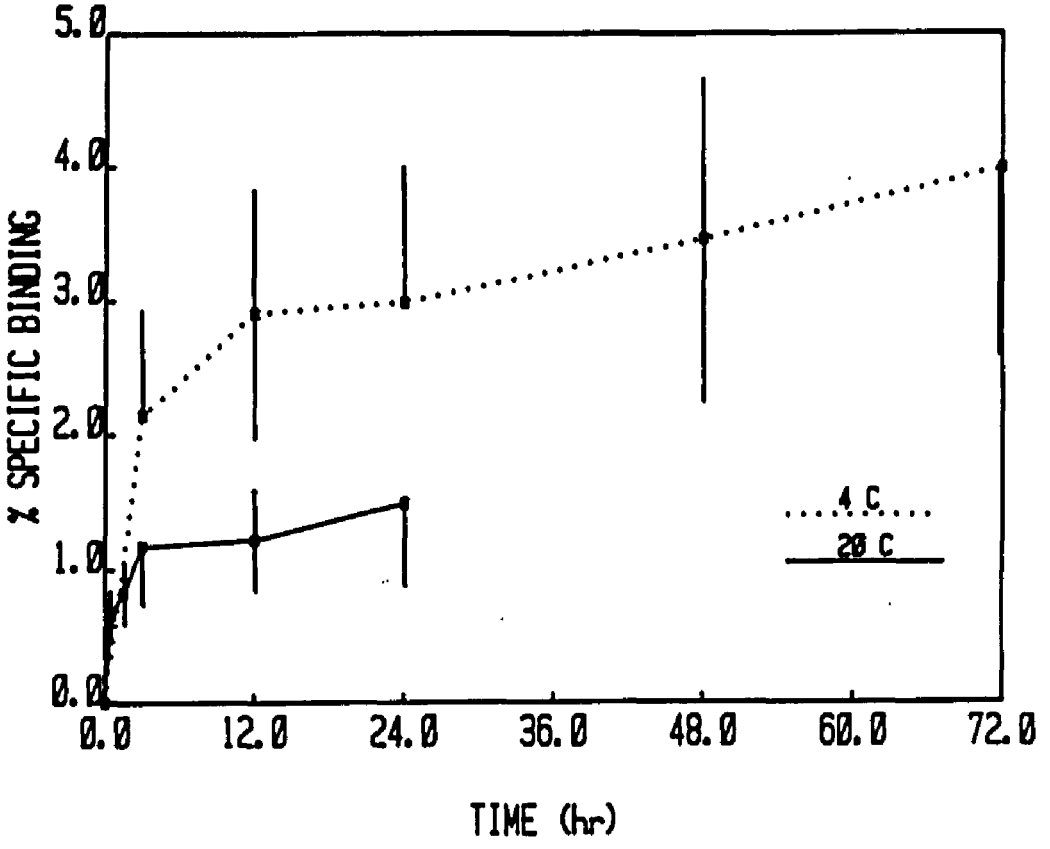

Figure 2. Time course of specific ^{125}I-insulin binding to bovine mammary microsomes. All incubations contained 500 µg Bradford membrane protein per ml. Each point represents the mean of triplicate determinations for six animals. Bars represent the SEM.

closely parallelled decreased binding at 20°C (Figure 3, panel B).

Increased insulin receptor degradation also may contribute to decreased insulin binding post-16 hr. Kahn et al (1974) demonstrated increased insulin receptor degradation at higher incubation temperatures. This would explain decreased insulin binding observed between 16 and 24 hr of incubation (17%) despite the maintenance of hormone integrity between these two times (93% TCA precipitability at 16 hr vs 92% TCA precipitability at 24 hr).

In contrast to liver microsomes (Figure 1), binding to mammary microsomes increased steadily over time at both 4° C and 20° C (Figure 2). For mammary microsomes of Figure 2, no insulin degradation was detected at either temperature over the time course of the study (Figure 3, panel A).

Less insulin bound to both liver and mammary preparations at the higher temperature. The specific binding of ^{125}I-insulin to liver microsomal membranes (500 µg Lowry protein/ml) at equilibrium was approximately 6.0% at 4°C, but only 3.7% at 20°C. Specific binding of ^{125}I-insulin to mammary microsomal membranes (500 µg Bradford protein/ml) at 24 hr of incubation was approximately 3.0% at 4°C and 1.5% at 20°C. Similar temperature effects on insulin binding have been demonstrated in erythrocytes (McElduff and Eastman, 1981) and lymphocytes (Gavin et al, 1973).

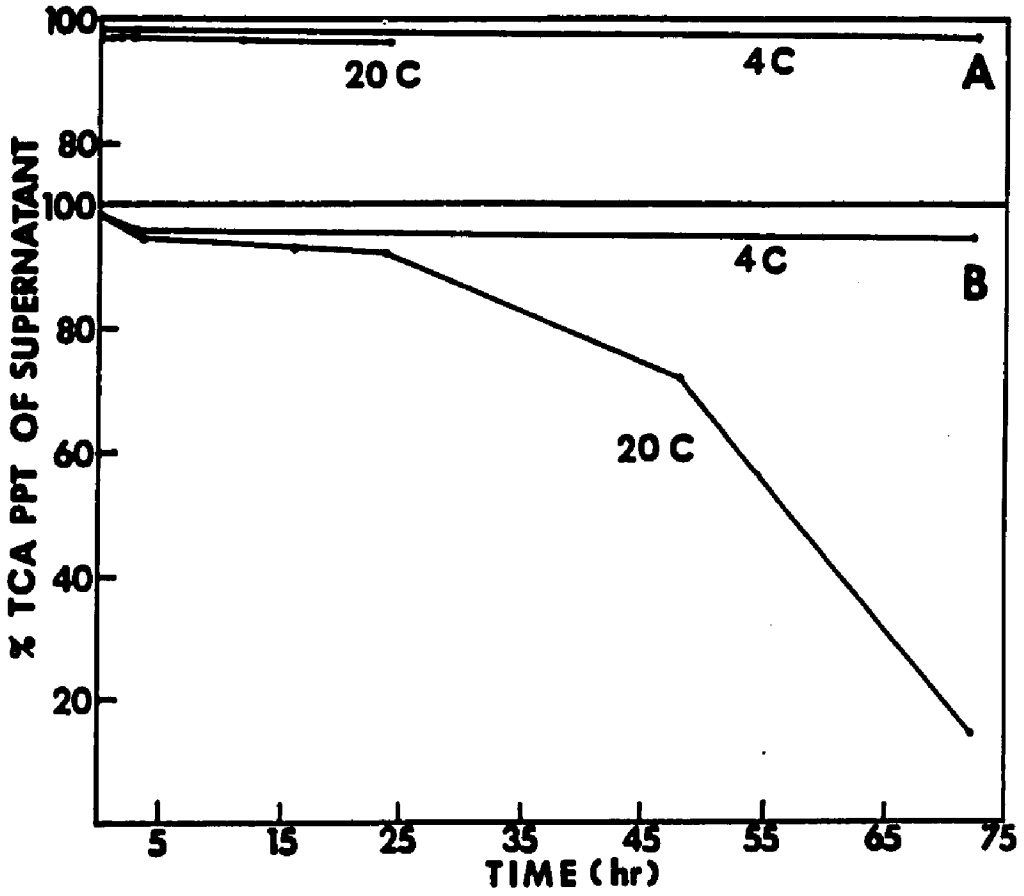

Figure 3. Time dependence of ^{125}I-insulin degradation by bovine liver (Panel B) and mammary (Panel A) microsomes. Data are the mean of six animals, except for 0.5, 1.5, and 3.0 hr data points at 20°C for bovine mammary microsomes (mean of four animals). Points on the Y axis represent TCA precipitability (PPT) of ^{125}I-insulin prior to incubation with microsomes. The average SE for the curves are as follows: liver, 4°C = 0.2; liver, 20°C = 2.1; mammary, 4°C = 0.1; and mammary, 20°C = 0.2.

Although decreased insulin binding at higher temperatures can be attributed partly to increased insulin and/or receptor degradation, a decrease in receptor binding affinity may also play an important role (Kahn et al, 1974). Olefsky and Ciaraldi (1981) suggested that in most receptor systems, dissociation rate is accelerated as a function of temperature to a greater degree than association rate, leading to an overall decrease in receptor affinity at higher temperatures.

Statistically, insulin binding to bovine mammary microsomes reached equilibrium by 3 hr of incubation at 4°C and 0.5 hr of incubation at 20°C (Figure 2). A comparison of insulin binding in bovine liver and mammary microsomes under similar incubation conditions (4° C, 48 hr) showed more variability among animals for binding to mammary microsomes than for liver microsomes. Specific ^{125}I-insulin binding to liver ranged from 5.61% - 9.96% with an average binding of 7.47% $\pm$ 0.68% (n=6 animals). Specific binding to mammary microsomes ranged from 0.11% - 2.44% with an average binding of 0.92% $\pm$ 0.34% (n=6 animals). The higher variation among animals of mammary microsomal insulin binding, as seen in Figure 2, may indicate a lack of equilibrium at either temperature. The binding profiles for mammary microsomes of individual cows showed abnormal patterns for several animals. Figure 4 is representative

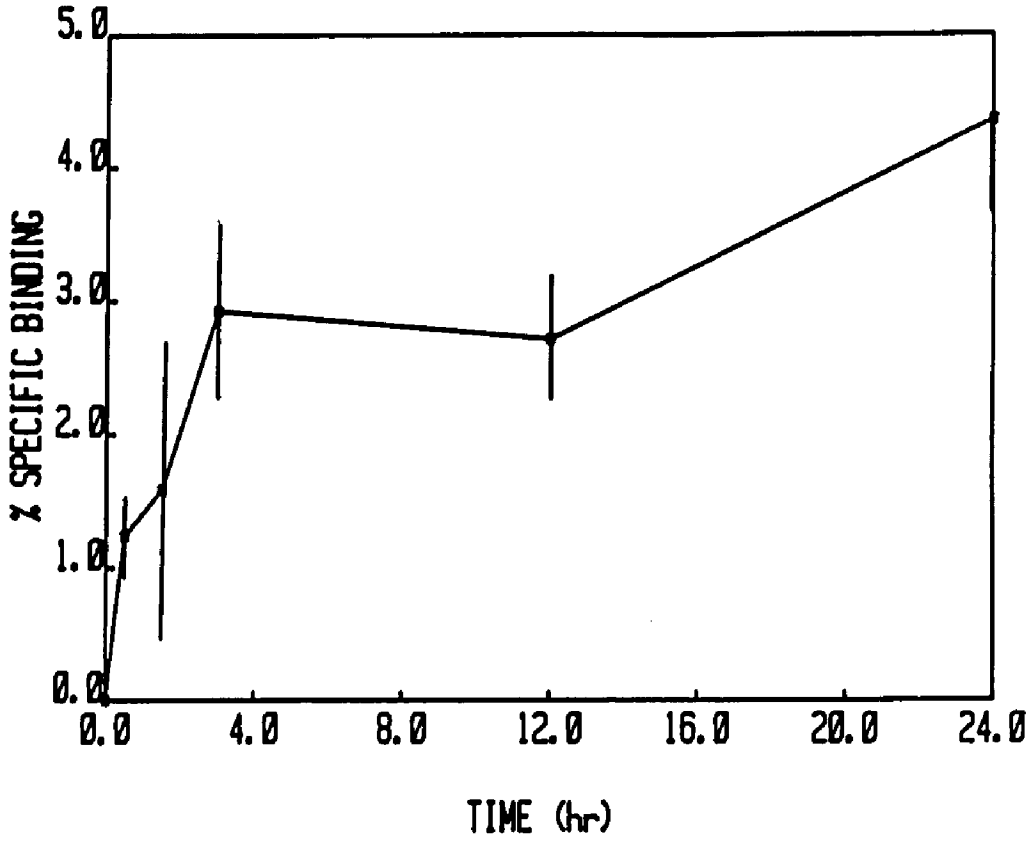

Figure 4. Time course of specific ^{125}I-insulin binding to Cow 2699 mammary microsomes. Each point represents the mean of triplicate determinations. Bars represent the standard deviation.

of the deviant binding behavior exhibited in these tissue preparations. Insulin binding appeared to reach steady state early in the incubation (in this case, 3 hr); however, continued sampling showed a further increase in binding at later times. This biphasic binding pattern suggested a dual population of insulin receptors in mammary microsomes; one population of receptors initially exposed to the incubation medium and another population of receptors exposed to the incubation medium at a later time.

One criterion of a hormone receptor is that the receptor must bind the hormone both rapidly and reversibly (Kahn, 1975). Mammary microsomal preparations in this study apparently failed to attain equilibrium at either temperature during the time course tested. Therefore, it is questionable whether the observed binding to bovine mammary microsomes meets the full criteria for a receptor.

The presence of cryptic receptors in bovine membranes could, however, explain the unusual binding behavior observed in mammary microsomal preparations in this study. Membrane alteration during the course of incubation could expose embedded receptors. The newly exposed receptors would require the same finite time to achieve equilibrium binding as the initially exposed receptors; however, they would attain equilibrium binding at a much later time during the binding assay than the initially exposed

population. If exposure of cryptic receptors occurred simultaneously, the binding curve should be characterized by two binding plateaus. The binding over time shown in Figure 4 for mammary microsomes suggests the presence of cryptic receptors which are exposed more or less simultaneously at a later time in the incubation.

The possibility of cryptic insulin receptors in bovine mammary microsomes was explored further with membrane perturbation studies. The results are in Figure 5. Inclusion of 2M NaCl in the radioreceptor assay buffer doubled insulin binding to rat liver microsomes (102% increase), but had relatively little effect on insulin binding to cow mammary microsomes (10% increase). Both enzymatic treatments of the membranes resulted in increased insulin binding to both rat liver and cow mammary microsomes. Phospholipase A_2 was more effective than was PL-C. Insulin binding increased 106% after PL-C digestion and 208% after PL-A_2 digestion in rat liver microsomes compared to increases of 43% and 123%, respectively, in bovine mammary microsomes. In general, all treatments were more effective in exposing binding sites in rat liver. Finally, the 37°C control tissues bound less insulin than the corresponding 20°C control microsomes indicating receptor degradation at the higher temperature.

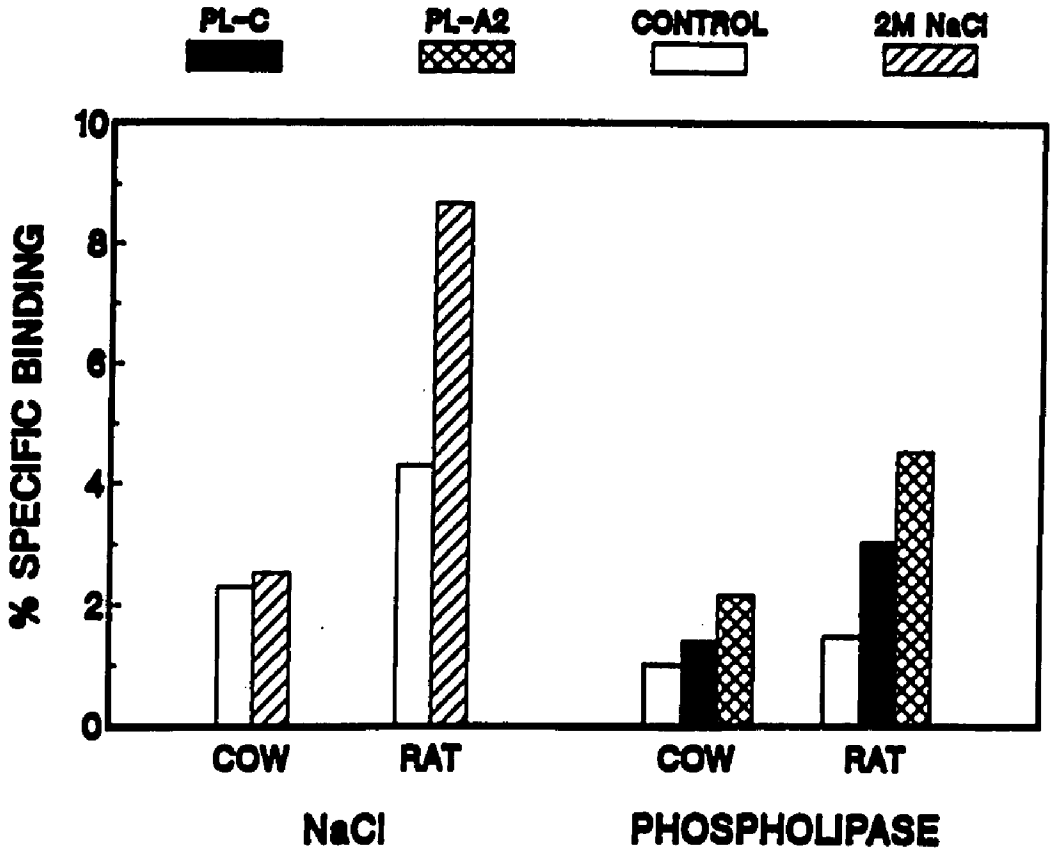

Figure 5. The effect of membrane perturbation on insulin binding to rat liver and cow mammary microsomes. All treatments are expressed as % specific binding per mg Lowry protein. NaCl Control = 20°C. Phospholipase Control = 37°C.

The results suggest the presence of cryptic receptors in rat liver and bovine mammary microsomes. Also, the different binding responses seen in these two tissues as a result of enzymatic and salt treatment show species differences in membrane organization and/or receptor exposure.

Since cryptic insulin binding sites appear to be present in bovine mammary microsomes, it is possible that spontaneous membrane restructuring and cryptic site exposure could occur upon prolonged in vitro incubation. If so, the bovine mammary insulin receptor would still attain equilibrium binding; however, the equilibrium as measured in this assay would be obscured by membrane changes during incubation, leading to continued exposure of new receptors not yet at equilibrium. To circumvent this difficulty, the time course of insulin binding to bovine mammary microsomes was examined post-PL-C digestion of the membranes. In this way, cryptic receptors would play a minimal role, most having been exposed at the start of the experiment.

Steady state binding in PL-C digested membranes was established by 3 hr of incubation at 20°C and maintained up to 12 hr of incubation (Figure 6). At 4°C, equilibrium was established statistically by 3 hr of incubation. As before, the large standard errors in the 4°C study reflect differences in binding among animals. Individual binding profiles showed that one of the animals examined at 4°C

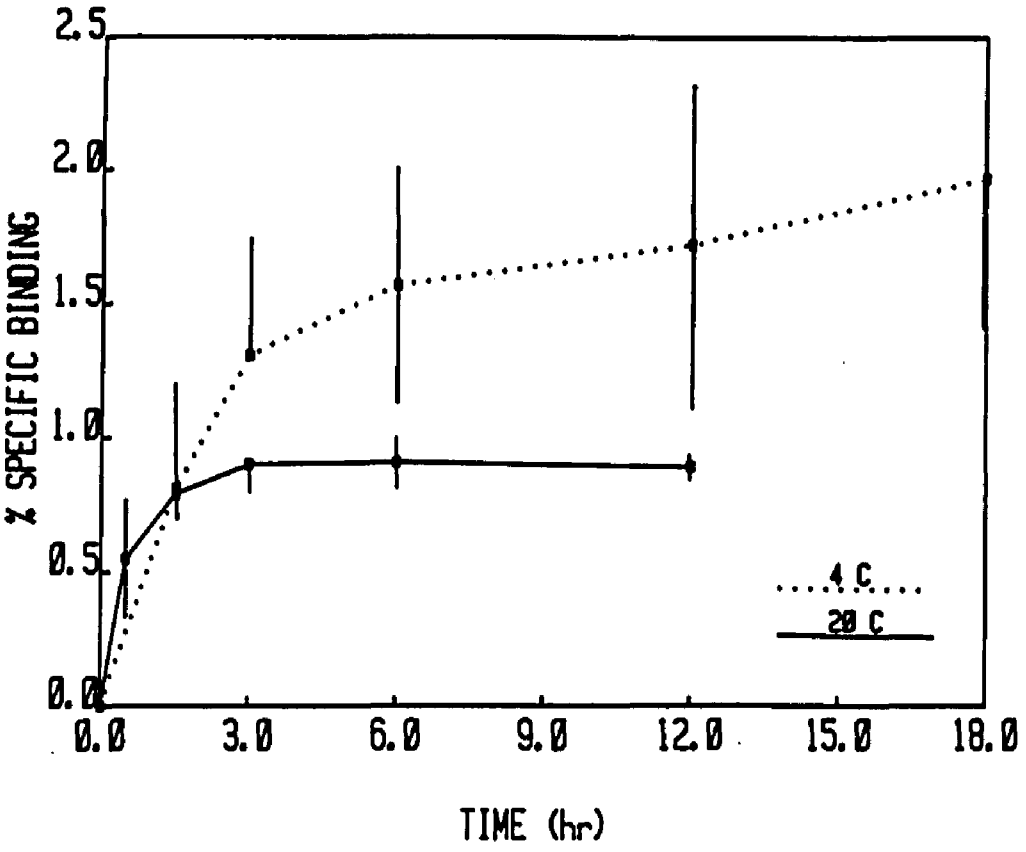

Figure 6. Time course of specific ^{125}I-insulin binding to bovine mammary microsomes post-PL-C digestion. Each point represents the mean of triplicate determinations for three animals. Bars represent SEM.

continued to exhibit a double plateau, in this case PL-C digestion failed to expose all cryptic receptors. The binding profiles ($4^{\circ}C$) from other animals in this study showed that steady state binding was attained by 12 hr at $4^{\circ}C$. The binding stability between 3 and 12 hr at $20^{\circ}C$ in all tissue preparations suggested that equilibrium had been attained within those times. Therefore, an assay time of 4.5 hr at $20^{\circ}C$ was adopted for the remainder of the mammary studies since equilibrium would be firmly established by this time, yet the chance of cryptic receptors being exposed would be negligible.

Figure 7 shows the effect of buffer pH on specific ^{125}I-insulin binding. Both bovine liver and mammary microsomes exhibited sharp pH optima of 7.8 and 8.0, respectively. A pH optimum of 7.5 - 8.0 has been reported for the insulin receptor from a variety of species and tissues including human lymphocytes (Gavin et al, 1973; Olefsky and Reaven, 1974), turkey erythrocytes (Ginsberg et al, 1977), rat mammary cells (Flint, 1982), rat adipocytes (Cuatrecasas, 1971b), and bovine liver plasma membranes (Rosen et al, 1979).

Hormone binding was specific for insulin in both liver and mammary microsomes. Insulin was approximately 12-fold more potent than proinsulin in displacing ^{125}I-insulin from bovine liver microsomes (Figure 8), while glucagon (200

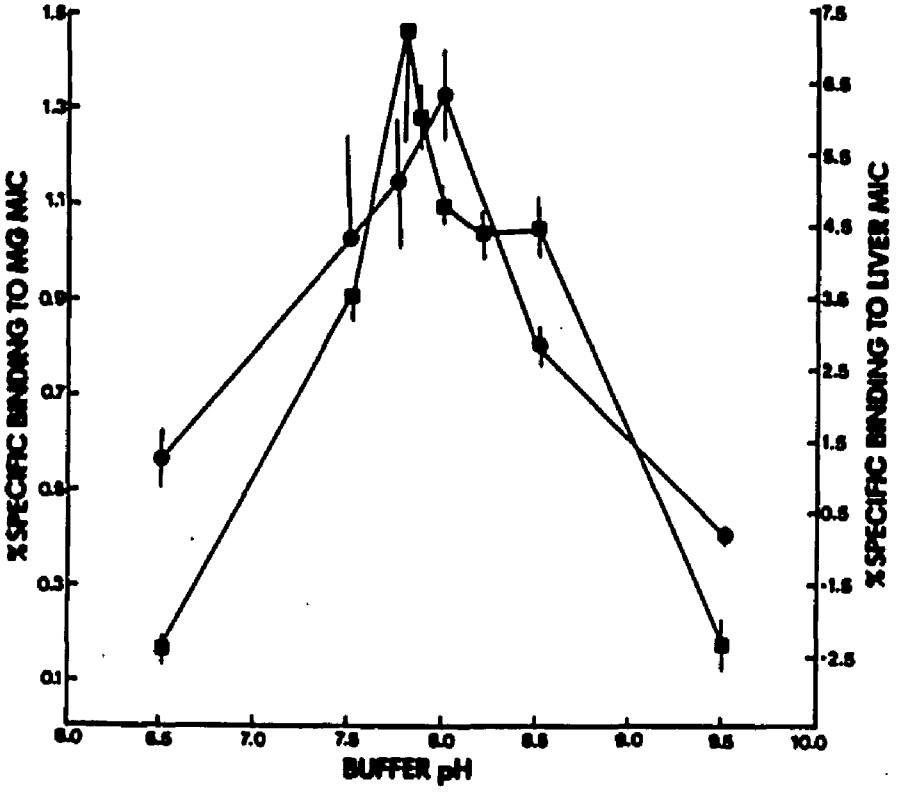

Figure 7. Specific ^{125}I-insulin binding to bovine liver and mammary microsomes as a function of buffer pH. Data are the mean ± SE for six animals (liver) (■) and the mean ± SE for two animals (mammary) (●).

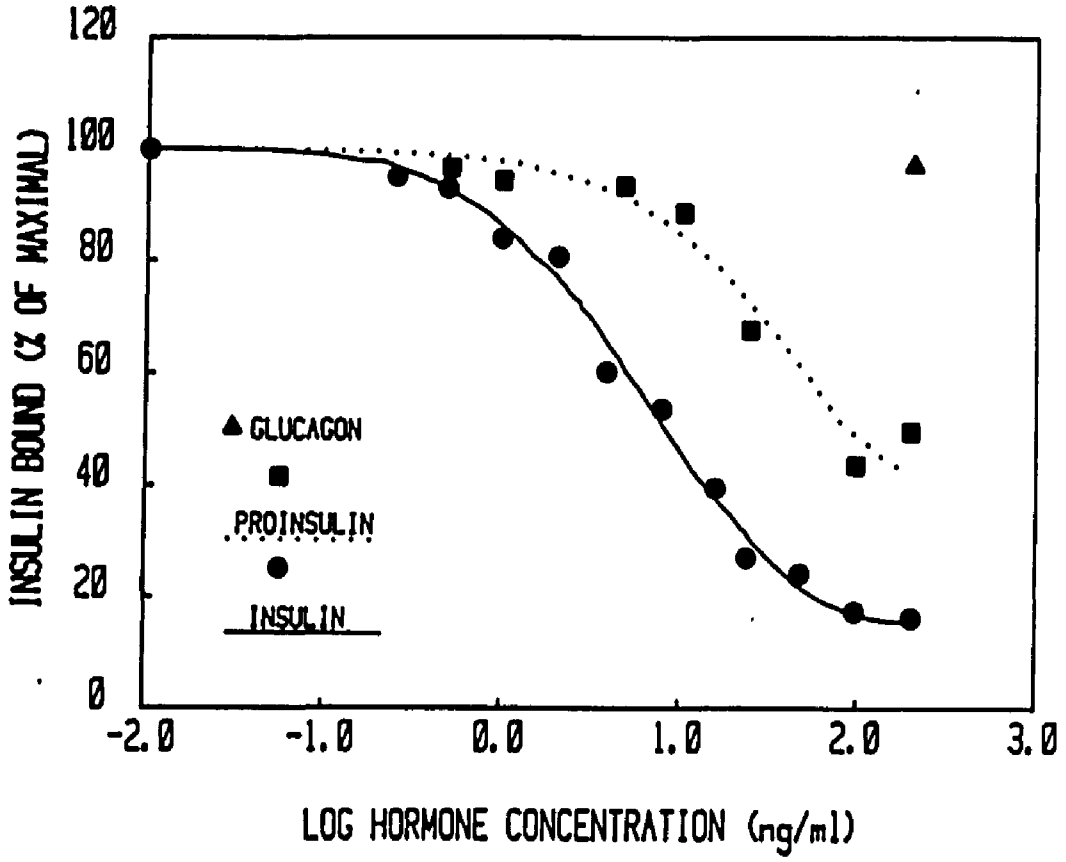

Figure 8. Specificity of the bovine liver insulin receptor. Data are the mean of triplicate determinations for six animals. Point on Y-axis represents ^{125}I-insulin binding at hormone concentration of 0.0 ng/ml. The average SE for the hormonal treatments are as follows: insulin = 2.5, proinsulin = 1.8, and glucagon = 2.2.

ng/ml) was ineffective in displacing insulin. Bovine mammary microsomes (Figure 9) demonstrated similar binding specificity with the exception that insulin was 34-fold more effective than proinsulin in competing for the insulin receptor.

Scatchard analyses of the competitive binding data for both bovine tissues yielded curvilinear plots (Figures 10 and 11). Such curvilinearity is typical of insulin binding data and is interpreted frequently as evidence for two insulin binding sites; a high affinity-low capacity site and a low affinity-high capacity site (Olefsky and Ciaraldi, 1981). However, DeMeyts et al (1976) have suggested that Scatchard curvilinearity signifies negative cooperativity among sites.

Since the explanation of curvilinearity remains debatable, the two receptor site model (Table 1) was chosen for analysis and description of the plots. The dissociation constants for both receptor populations in bovine mammary microsomes and for the high affinity receptor population in bovine liver microsomes are similar to those reported previously for a variety of tissues and species (Kahn et al, 1974; Olefsky and Reaven, 1974; Etherton and Walker, 1982; Inagaki and Kohmoto, 1982; Haskell et al, 1984). On the other hand, the K_d value obtained for the low affinity-high capacity binding site of bovine liver (K_d

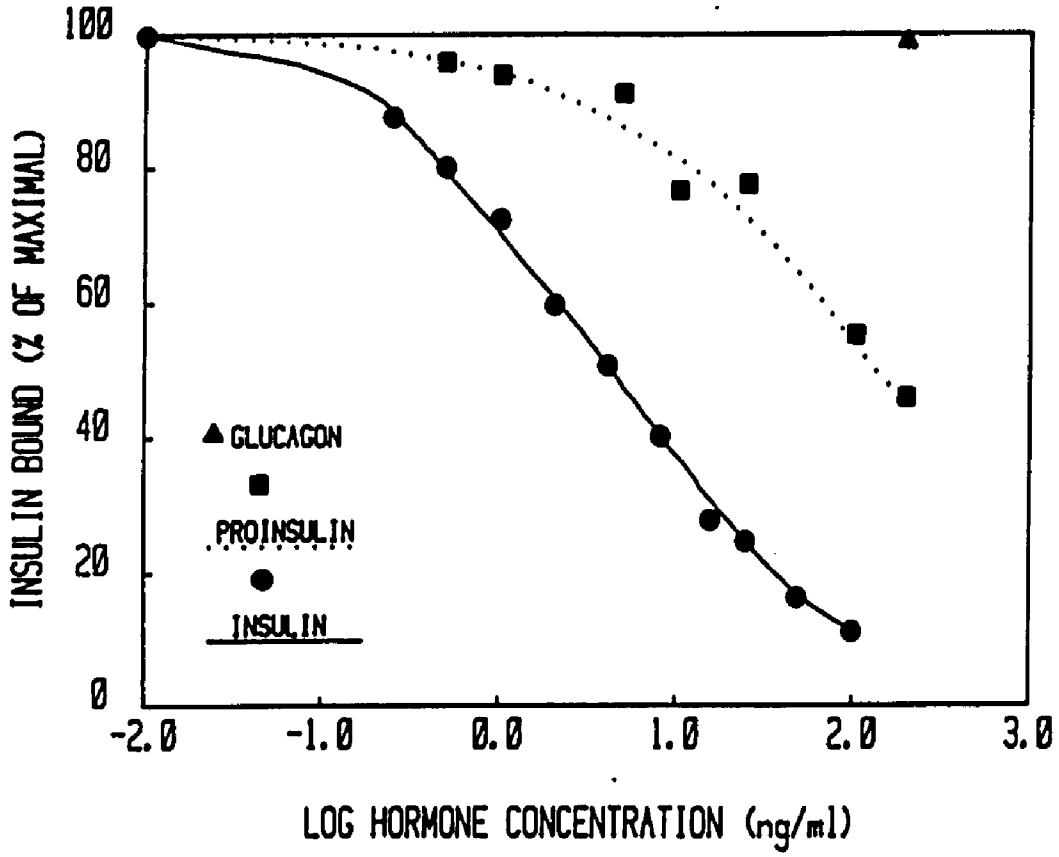

Figure 9. Specificity of the bovine mammary insulin receptor. Data are the mean of triplicate determinations for two animals. Point on the Y-axis represents ^{125}I-insulin binding at a hormone concentration of 0.0 ng/ml. The average SE for the hormonal treatments are as follows: insulin = 1.5, proinsulin = 1.2, and glucagon = 1.4.

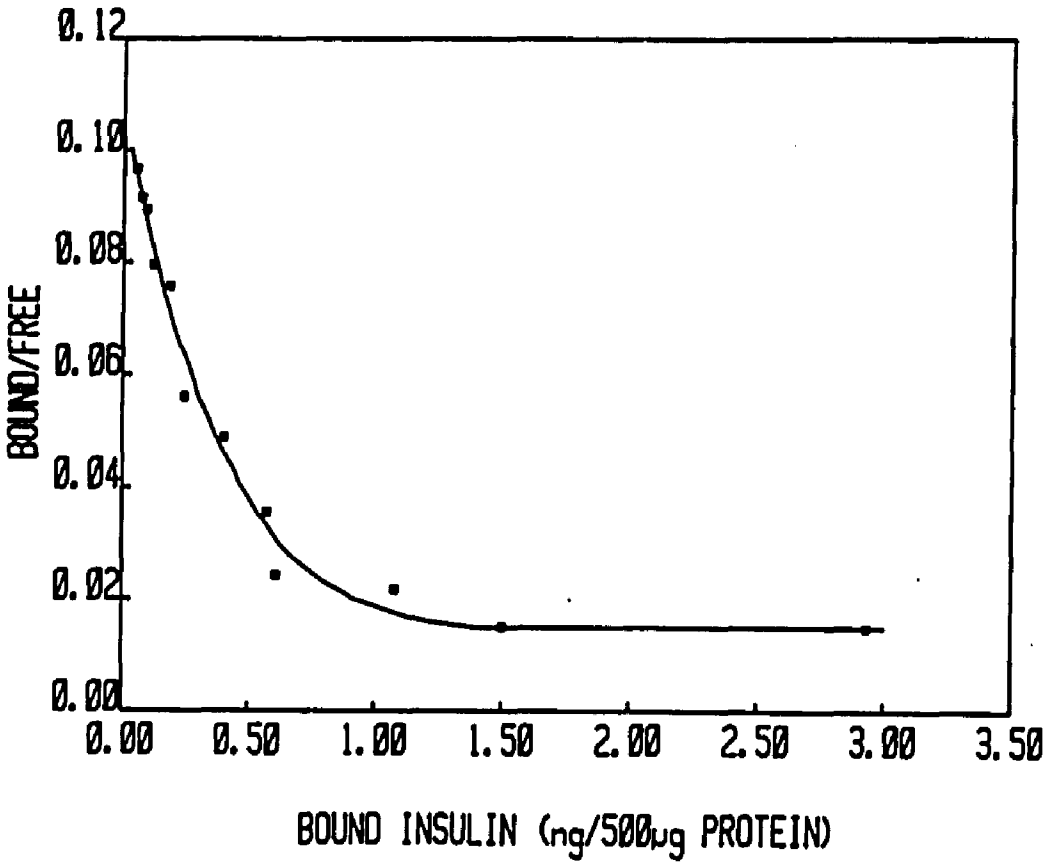

Figure 10. Scatchard plot of the competitive binding data of bovine liver microsomes presented in Figure 8. Each point represents the mean of triplicate determinations for six animals.

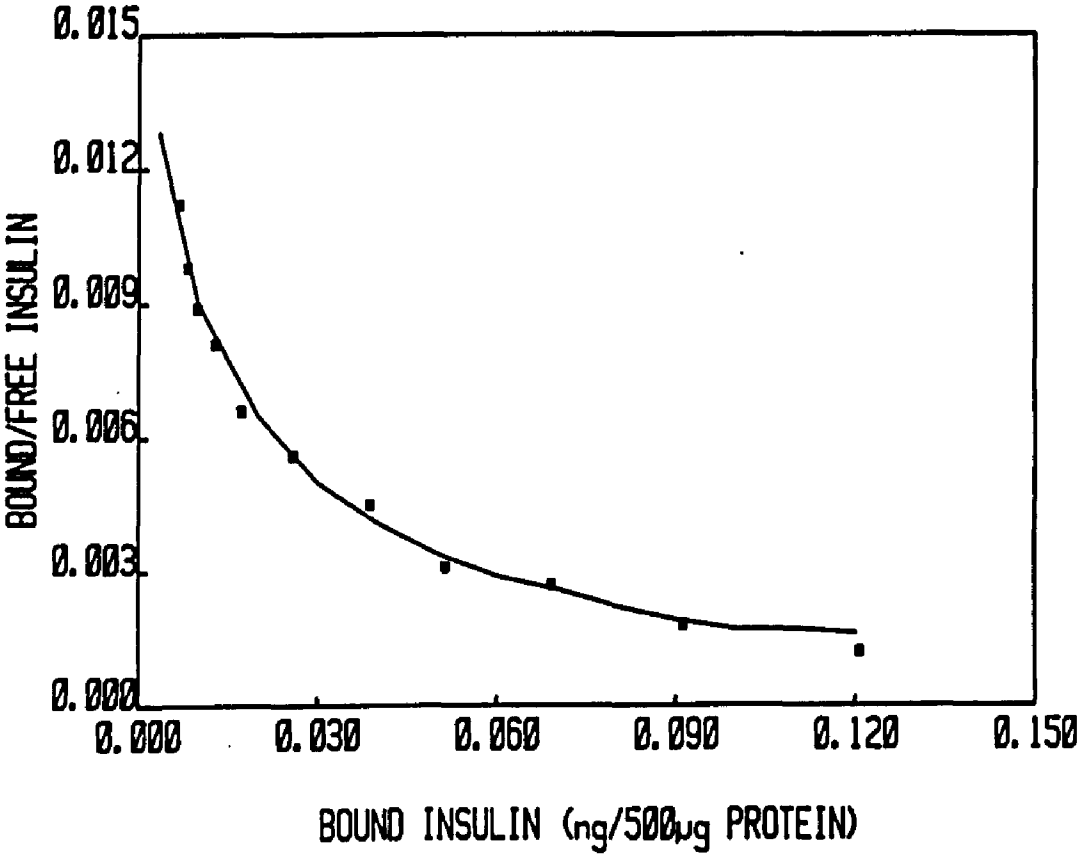

Figure 11. Scatchard plot of the competitive binding data of bovine mammary microsomes presented in Figure 9. Each point represents the mean of triplicate determinations for two animals.

Table 1. Binding capacities and apparent dissociation constants for bovine insulin of microsomal membranes from bovine liver and mammary tissue.

	Membrane Source	
Apparent Dissociation Constant K_d (M)	Liver[#]	Mammary Gland[##]
High Affinity Binding Site	7.6×10^{-10}	9.6×10^{-11}
SE	1.1×10^{-10}	7.5×10^{-11}
Low Affinity Binding Site	3.2×10^{-6}	6.8×10^{-9}
SE	1.3×10^{-6}	4.5×10^{-9}
Binding Capacity (moles/mg)		
High Affinity Binding Site	1.4×10^{-13}	2.8×10^{-15}
SE	2.0×10^{-14}	1.9×10^{-15}
Low Affinity Binding Site	5.4×10^{-11}	5.2×10^{-14}
SE	2.5×10^{-11}	2.3×10^{-14}

[#] n=6
[##] n=2
* Moles hormone bound per mg Lowry microsomal protein.

= 3.2×10^{-6}M) is several orders of magnitude less than those cited previously and may be attributable to a lack of data points in the lower portion of the Scatchard plots for bovine liver.

Incubation conditions for the competitive binding assays differed. Bovine liver was incubated at 4°C for 48 hr while bovine mammary tissue was incubated at 20°C for 4.5 hr. The lower temperature was adopted for the standard liver radioreceptor assay because insulin binding was greater at this temperature, therefore increasing the sensitivity of the assay. A lower incubation temperature also would have been desirable for the mammary radioreceptor assay because mammary microsomes exhibited low insulin binding. However, their aberrant binding behavior over time at 4° C obscured steady state binding at this temperature, even after enzymatic treatment of the membranes. Since the validity of Scatchard analysis depends on the establishment of equilibrium binding in the assay, the higher temperature was employed for the mammary assay.

Incubation temperature may affect receptor binding affinity by altering the dissociation rate constant of the receptor; hence, a direct comparison of the binding affinities of bovine liver and mammary tissue cannot be made. Nevertheless, the insulin receptor of bovine mammary tissue has a higher affinity for insulin than does the receptor of

bovine liver since the K_d of liver was lower than the K_d of mammary even at the lower temperature.

The binding capacities of both the high and low affinity sites of bovine liver microsomes observed in this study (Table 1) are very similar to those reported by Rosen et al (1979) for bovine liver plasma membranes (R_1 = 2.7 x 10^{-13} moles/mg protein; R_2 = 1.4 x 10^{-12} moles/mg protein). However, neither our study nor the Rosen et al (1979) study tested for cryptic receptors in this tissue. Therefore, the binding capacities of both sites reported in this paper and that of Rosen et al (1979) may not be a true reflection of effective receptor concentration in bovine liver.

Estimates of insulin receptor loading in bovine liver and mammary tissue would provide information concerning receptor regulation and perhaps tissue responsiveness. Such estimates can be made using the data in Table 1 and the mass action expression for hormone receptor binding at equilibrium:

$$[IR] = \frac{[I][R_T]}{K_d + [I]}$$

where [I] = free insulin concentration, $[R_T]$ = total insulin receptor concentration, [IR] = occupied insulin receptor concentration, and K_d = dissociation constant (Olefsky and Ciaraldi, 1981). Plasma insulin concentrations, available from other studies in our

laboratory, of 1.0×10^{-10}M to 1.7×10^{-10}M (15-25 μU/ml) are characteristic of cattle receiving a typical 60:40 roughage:concentrate diet. Using an average plasma insulin concentration of 20 μU/ml or 1.4×10^{-10}M and the values for K_d and $[R_T]$ in Table 1, the degree of hormone loading for both bovine liver and mammary microsomes can be calculated. Since the biological significance of the low affinity receptor sites is uncertain, (Cuatrecasas and Hollenberg, 1976), only high affinity sites are considered here. For bovine liver microsomes, the concentration of insulin-bound receptors is 1.1×10^{-11}M or 15% of the total receptors present. In the case of bovine mammary microsomal receptors, 8.3×10^{-13}M are occupied or 59% of the total.

It appears from previous studies that only a fraction of the total hormone receptors present on a cell must be occupied to elicit a maximal cellular response (Olefsky and Ciaraldi, 1981). Maximum glucose oxidation in rat adipocytes was attained with only 2.4% loading of insulin receptors (Kono and Barham, 1971). Olefsky (1975) noted that maximal stimulation of glucose uptake by adipocytes occurred when only 10% of the insulin receptors were filled.

From this, both bovine liver and mammary tissue ought to be under maximal insulin stimulation at plasma insulin

concentrations of $\geq$ 9.0 x 10^{-11}M (13 μU/ml); and refractory to greater stimulation by higher insulin concentrations. If 10% receptor occupancy is accepted as the degree of receptor loading required to elicit a maximal intracellular response, a plasma insulin concentration as low as 3.3 x 10^{-11}M (0.2 ng/ml) would still result in maximal stimulation of bovine mammary tissue (ca 26% receptor occupancy). Only at insulin concentrations far below 3.3 x 10^{-11}M would the insulin response in bovine mammary tissue be diminished. Since 3.3 x 10^{-11}M is the lowest plasma insulin concentration documented in ruminants (Horino et al, 1968; McAtee and Trenkle, 1971), bovine mammary tissue would probably be in a constant state of maximal insulin stimulation at a constant $[R_T]$ and K_d.

This agrees with other studies wherein it appears that the mammary gland is insulin-independent with respect to glucose uptake (Martin and Baldwin, 1971; Hove, 1978). However, Threadgold and Kuhn (1984) recently reported that insulin promoted 3-0-methyl glucose transport into the mammary gland of starved, but not fed, rats. These results suggest that insulin is required for glucose transport in nonruminant mammary tissue, but that a response is not apparent because insulin stimulation of transport is maximal under most physiological conditions. Extreme conditions, such as starvation, could cause plasma insulin to fall

below the concentration required for maximum stimulation of mammary insulin receptors. In this case, glucose transport by the mammary gland would be a function of the insulin concentration. If our calculations are representative, the plasma insulin concentration in the ruminant would have little or no influence in regulating cellular insulin response of the mammary gland at the receptor level under physiological conditions. This could be a metabolic adaptation of the gland to ensure a constant supply of glucose; hence, preserving milk secretion under extreme physiological conditions.

Not all insulin-dependent cellular functions will necessarily conform to the spare receptor theory. Dolais-Kitabgi et al (1981) reported that insulin stimulation of amino acid transport into rat hepatocytes was in direct proportion to insulin binding up to 80% receptor occupancy. Therefore, it is possible that while some cellular mechanisms seem to require threshold concentrations of insulin for maximal stimulation, others respond to insulin in a dose-dependent fashion. Recently, Vernon et al (1985) examined insulin action and binding in bovine adipocytes maintained in vitro. Half maximal stimulation of both glucose transport and pyruvate kinase activity were observed at an insulin concentration of 8.3×10^{-11}M (0.5 ng/ml). However, calculations based on their insulin

binding data approximate 17% receptor occupancy at this insulin concentration. This is slightly higher than for rat adipocytes (Olefsky, 1975) where maximal stumulation of glucose transport occurred at 10% insulin receptor occupancy. Determination of a regulatory role for the insulin receptor in bovine tissues depends on identification of those cellular mechanisms which are insulin dose-dependent.

In conclusion, the specificity and kinetics of ^{125}I-insulin binding to bovine mammary microsomes indicate the presence of insulin receptors in bovine mammary tissue. These receptors are similar in binding affinity and pH optimum to insulin receptors in other species and tissues, including bovine liver.

Both bovine mammary and rat liver microsomes demonstrated increased ^{125}I-insulin binding after membrane perturbation with phospholipases, suggesting the existence of cryptic receptors in both microsomal preparations. The response to enzyme perturbation was more pronounced in rat liver than in bovine mammary microsomes, and only rat liver microsomes responded to NaCl treatment.

The reason for these species differences in insulin binding, both pre- and post-membrane perturbation, is not apparent, but may reflect differences in fatty acid composition of membrane phospholipids between ruminants and nonruminants. It is well documented that depot fat of

ruminants is composed of more saturated and more trans fatty acids than depot fat of nonruminants (Shorland, 1953; Hartman et al, 1954). This difference in fat composition results from microbial biohydrogenation of dietary fat in the rumen (Dawson and Kemp, 1970). Although differences in membrane lipid composition between ruminants and non-ruminants have not been documented, a higher percentage of saturated and trans fatty acids in ruminant membranes could decrease membrane fluidity (Wahle, 1983), and thereby influence insulin receptor binding (Berlin et al, 1985).

Although the microsomal preparations employed in this study provided a relatively easy and inexpensive means of evaluating insulin receptors in bovine mammary tissue, extrapolation of our results to regulation in the intact cell may not be appropriate. Further studies correlating insulin binding and biological response in bovine mammary secretory cells or alveoli are indicated. Use of viable tissue preparations would provide more accurate information as to the relationship between insulin binding and action in the ruminant mammary gland.

LIST OF REFERENCES

Bergman, E. N., Katz, M. L., and Kaufman, C. F. (1970). Quantitative aspects of hepatic and portal glucose metabolism and turnover in sheep. Am. J. Physiol. 219, 785-793.

Berlin, E., Bhathena, S. J., Kliman, P. G., and Revett, K. (1985). Effect of saturation of dietary lipids on insulin receptors and membrane fluidity in rabbits. Fed. Proc. Abstr. 44, 1236.

Bolander, F. F., Jr., Nicholas, K. R., Van Wyk, J. J., and Topper, Y. J. (1981). Insulin is essential for accumulation of casein mRNA in mouse mammary epithelial cells. Proc. Natl. Acad. Sci. USA 78, 5682-5684.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Cuatrecasas, P. (1971a). Unmasking of insulin receptors in fat cells and fat cell membranes. J. Biol. Chem. 246, 6532-6542.

Cuatrecasas, P. (1971b). Properties of the insulin receptor of isolated fat cell membranes. J. Biol. Chem. 246, 7265-7274.

Cuatrecasas, P., Desbuquois, B., and Krug, F. (1971). Insulin-receptor interactions in liver cell membranes. Biochem. Biophys. Res. Commun. 44, 333-339.

Cuatrecasas, P. and Hollenberg, M. D. (1976). Membrane receptors and hormone action. In Advances in Protein Chemistry (Edited by Anfinsen, C. B., Edsall, J. T., and Richards, F. M.) pp. 251-451. Academic Press, New York.

Dawson, R. M. C. and Kemp, P. (1970). Biohydrogenation of dietary fats in ruminants. In Physiology of Digestion and Metabolism in the Ruminant (Edited by Phillipson,

A. T.) pp. 504-518. Oriel Press, Newcastle upon Tyne.

DeMeyts, P. A., Bianco, A. R., and Roth, J. (1976). Site - site interactions among insulin receptors. Characterization of the negative cooperativity. J. Biol. Chem. 251, 1877-1887.

Dolais-Kitabgi, J., Checler, F., Rey, J., Morin, O., and Freychet, P. (1981). Insulin stimulation of amino acid transport in primary cultured rat hepatocytes varies in direct proportion to insulin binding. FEBS Letters 128, 321-324.

Etherton, T. D. and Walker, O. A. (1982). Characterization of insulin binding to isolated swine adipocytes. Endocrinology 110, 1720-1724.

Flint, D. J. (1982). Insulin binding to rat mammary gland at various stages of cell isolation and purification. Mol. Cell. Endocrinol. 26, 281-294.

Freychet, P., Kahn, R., Roth, J., and Neville, D. M., Jr., (1972). Insulin interactions with liver plasma membranes: Independence of binding of the hormone and its degradation. J. Biol. Chem. 247, 3953-3961.

Freychet, P., Roth, J., and Neville, D. M., Jr., (1971). Monoiodoinsulin: Demonstration of its biological activity and binding to fat cells and liver membranes. Biochem. Biophys. Res. Commun. 43, 400-408.

Gavin, J. R. III, Gorden, P., Roth, J., Archer, J. A., and Buell, D. N. (1973). Characteristics of the human lymphocyte insulin receptor. J. Biol. Chem. 248, 2202-2207.

Gill, R. D. and Hart, I. C. (1980). Properties of insulin and glucagon receptors on sheep hepatocytes: A comparison of hormone binding and plasma hormones and metabolites in lactating and non-lactating ewes. J. Endocrinol. 84, 237-247.

Ginsberg, B. H., Kahn, C. R., and Roth, J. (1977). The insulin receptor of the turkey erythrocyte: Similarity to mammalian insulin receptors. Endocrinology 100, 82-90.

Grizard, J. (1983). Insulin binding to skeletal muscle membranes in growing ruminating sheep fed different diets. Reprod. Nutr. Develop. 23, 389-401.

Hartman, L., Shorland, F. B., and McDonald, I. R. C. (1954). Occurrence of trans-acids in animal fats. Nature 174, 185-186.

Haskell, J. F., Meezan, E., and Pillion, D. J. (1984). Identification and charcterization of the insulin receptor of bovine retinal microvessels. Endocrinolgy 115, 698-704.

Horino, M., Machlin, L. J., Hertelendy, F., and Kipnis, D. M. (1968). Effect of short-chain fatty acids on plasma insulin in ruminant and nonruminant species. Endocrinology 83, 118-128.

Hove, K. (1978). Effects of hyperinsulinemia on lactose secretion and glucose uptake by the goat mammary gland. Acta Physiol. Scand. 104, 422-430.

Inagaki, Y. and Kohmoto, K. (1982). Changes in Scatchard plots for insulin binding to mammary epithelial cells from cycling, pregnant, and lactating mice. Endocrinology 110, 176-182.

Khan, C. R. (1975). Membrane receptors for polypeptide hormones. In Methods in Membrane Biology. (Edited by Korn, E. D.) pp. 81-146. Plenum Publishing, New York.

Khan, R. C., Freychet, P., Roth, J., and Neville, D. M., Jr. (1974). Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. J. Biol. Chem. 249, 2249-2257.

Kono, T. and Barham, F. W. (1971). The relationship between the insulin-binding capacity of fat cells and the cellular response to insulin. Studies with intact and trypsin-treated fat cells. J. Biol. Chem. 246, 6210-6216.

Kulski, J. K., Nicholas, K. R., Topper, Y. J. and Qasba, P. (1983). Essentiality of insulin and prolactin for accumulation of rat casein mRNAs. Biochem. Biophys. Res. Commun. 116, 994-999.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Martin, R. J. and Baldwin, R. L. (1971). Effects of insulin and anti-insulin serum treatments on levels of

metabolites in rat mammary glands. Endocrinology 88, 868-871.

McAtee, J. W. and Trenkle, A. (1971). Metabolic regulation of plasma insulin levels in cattle. J. Anim. Sci. 33, 438-442.

McElduff, A. and Eastman, C. J. (1981). The erythrocyte insulin receptor. Aust. J. Exper. Biol. Med. Sci. 59, 439-448.

Metzler, C. M., Elfring, G. L., McEwen, A. J. (1974). A package of computer programs for pharmaco-kinetic modeling. Biometrics 30, 562-563.

Nicholas, K. R. and Topper, Y. J. (1983). Anti-insulin receptor serum mimics the developmental role of insulin in mouse mammary explants. Biochem. Biophys. Res. Commun. 111, 988-993.

Nowak, J. and Dzialoszynski, L. (1967). Effect of experimental alloxan diabetes on the secretion and composition of goat milk. Acta Physiol. Pol. 18, 595-606.

Olefsky, J. M. (1975). Effect of dexamethasone on insulin binding, glucose transport, and glucose oxidation of isolated rat adipocytes. J. Clin. Invest. 56, 1499-1508.

Olefsky, J. M. and Ciaraldi, T. P. (1981). The insulin receptor: Basic characteristics and its role in insulin resistant states. In Diabetes Mellitus. (Edited by Brownlee, M.) pp. 73-116. Garland STPM Press, New York.

Olefsky, J. and Reaven, G. M. (1974). The human lymphocyte: A model for the study of insulin-receptor interaction. J. Clin. Endocrinol. Metab. 38, 554-560.

Peacock, M. L., Bar, R. S., and Goldsmith, J. (1982). Interactions of insulin with bovine endothelium. Metabolism 31, 52-56.

Pocius, P. A., Dreels, J. M., Devery-Pocius, J. E., and Baumrucker, C. R. (1984). Techniques for preparation of bovine mammary smooth membranes. J. Dairy Sci. 67, 2055-2061.

Rosen, P., Ehrich, B., Junger, E., Bubenzer, H. J., and Kuhn, L. (1979). Binding and degradation of insulin by plasma membranes from bovine liver isolated by large scale preparation. Biochim. Biophys. Acta 587, 593-605.

Rutter, W. J., Pictet, R. L., and Morris, P. W. (1973). Toward molecular mechanisms of developmental processes. In Annual Review of Biochemistry (Edited by Snell, E. E., Boyer, P. D., Meister, A., and Richardson, C. C.) pp. 601-646. Annual Reviews, Inc., Palo Alto.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. 51, 660-672.

Schacterle, G. R. and Pollack, R. L. (1973). A simplified method for the quantitative assay of small amounts of protein in biologic material. Anal. Bioch. 51, 654-655.

Shorland, F. B. (1953). Animal fats: Recent researches in the Fats Research Laboratory, D.S.I.R., New Zealand. J. Sci. Food Agric. 4, 497-503.

Threadgold, L. C. and Kuhn, N. J. (1984). Monosaccharide transport in the mammary gland of the intact lactating rat. Biochem. J. 218, 213-219.

Topper, Y. J. and Freeman, C. S. (1980). Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev. 60, 1049-1106.

Trenkle, A. (1972). Radioimmunoassay of plasma hormones: Review of plasma insulin in ruminants. J. Dairy Sci. 55, 1200-1211.

Vernon, R. G., Finley, E., Taylor, E., Flint, D. J. (1985). Insulin binding and action on bovine adipocytes. Endocrinology 116, 1195-1199.

Wahle, K. W. J. (1983). Fatty acid modification and membrane lipids. Proc. Nutr. Soc. 42, 273-287.

CHAPTER III

Insulin Binding by Bovine and Porcine Membranes

INTRODUCTION

Both bovine liver and mammary microsomes have high affinity insulin receptors with affinity constants of 1.3 x $10^{9}M^{-1}$ and 1.0 x $10^{10}M^{-1}$, respectively (Smith et al., 1986). These constants are similar to those for high affinity insulin binding by nonruminant tissues (1.1 x $10^{8}M^{-1}$ to 2.0 x $10^{10}M^{-1}$) (Kahn, 1975).

Although mammary microsomes may be used to assess insulin receptors in the mammary gland (Smith et al., 1986), the membrane source must be obtained by mammary biopsy or slaughter. In contrast, milk is easily obtained from the mammary gland and contains abundant membranes of mammary secretory cell origin.

Fat droplets are enveloped in a portion of the apical plasma membrane of the mammary epithelial cell during the secretory process and occur in milk as membrane-bounded fat droplets termed milk fat globules (MFG) (Mather & Keenan, 1983). The milk fat globule membranes (MFGM) (Mather

& Keenan, 1983) share many properties with the apical mammary plasmalemma, are easily isolated from milk (Dowben et al., 1967) and contain numerous proteins, such as HLA antigens (Wiman et al., 1979), xanthine oxidase, and butyrophilin (Mather & Keenan, 1983). Flint and West (1983) examined ^{125}I-insulin binding to caprine and murine MFGM in terms of hormone specificity and pH optimum. They concluded that insulin receptors were present in MFGM but did not describe the amount of specific ^{125}I-insulin binding observed. Since insulin receptors appear to be present in MFGM, we postulated that MFGM could be a more convenient system than mammary microsomes for monitoring insulin receptor regulation in the mammary gland of lactating animals.

While the affinity constants for bovine liver and mammary insulin receptors we reported were similar to each other, the binding capacities differed by 50-fold (1.4×10^{-13} vs 2.8×10^{-15} moles insulin bound/mg microsomal protein, respectively) (Smith et al., 1986). Insulin binding by microsomal preparations has been shown to vary with species and tissue examined (Posner et al., 1974) which may indicate differential insulin requirements both among species and among tissues within species. In particular, substantial differences in insulin binding might

be expected between ruminants and nonruminants, as glucose metabolism is known to differ (Lindsay, 1978).

Since little glucose is absorbed from the gut of ruminants (Lindsay, 1978), endogenous glucose production is necessary to meet their glucose requirement. Gluconeogenesis appears to be permanently active in the ruminant (Lindsay, 1978), occurring primarily in the liver (Bergman et al., 1974). Both blood glucose and insulin concentrations are lower in ruminants (Horino et al., 1968) than in nonruminants (Yalow & Berson, 1960) (fasting glucose = 50 vs 90 mg/dl, respectively; fasting insulin = 3×10^{-11}M vs 1.5×10^{-10}M, respectively). Furthermore, ruminants seem less sensitive to insulin than nonruminants. In diabetic sheep, blood glucose concentrations respond slowly (4 days) to intravenous administration of insulin (Jarrett et al., 1974). In diabetic nonruminants, the same response occurs with 30 min (Ganong, 1975).

Based on differences between ruminants and nonruminants in glucose metabolism and insulin regulation thereof (Jarrett et al., 1974), we postulated that similar differences might exist in insulin binding between these two animal groups.

Two studies were conducted to test our postulates. In Study 1, insulin binding to bovine MFGM was measured and compared to insulin binding by bovine mammary microsomes. In Study 2, insulin binding to liver and mammary microsomes of the pig and dairy cow, species not examined by Posner et al. (1974), was examined and compared.

MATERIALS AND METHODS

Carrier-free Na ^{125}I (17.4 Ci/mg) was obtained from New England Nuclear Products (Boston, MA, U.S.A.). Bovine serum albumin (BSA, fraction V) and guinea pig antibovine insulin serum (GPAIS) were purchased from Miles Biochemical (Elkhart, IN, U.S.A.). Bovine crystalline insulin (26.6 units/mg) was generously provided by W. Fields of the Eli Lilly Company (Indianapolis, IN, U.S.A.).

Mammary tissue was obtained from three lactating crossbred sows (21-28 days lactation) and six lactating Holstein cows (77-390 days lactation). Liver was obtained from one lactating, crossbred sow (28 days lactation), three lactating Holstein cows (270-437 days lactation), and three nonlactating cows (two Holstein and one Jersey). All tissues were removed immediately postslaughter and

held in ice until processing. Milk samples (AM) were collected from each of the lactating cows within 4 days prior to their slaughter for mammary tissue.

Liver and mammary microsome preparation was as described previously (Smith et al., 1986). Crude MFGM were prepared according to Dowben et al. (1967) except that all wash steps were executed at 20°C rather than at 35°C to retard insulin receptor degradation. The final microsomal and MFGM pellets were resuspended in 50 mM Tris/HCl, 10 mM $CaCl_2$, pH 7.5 at 4°C. Membrane protein content was measured by the Lowry method (1951) with BSA as a standard. The freshly isolated bovine mammary microsomes and MFGM were then assayed for insulin binding, and the remaining bovine mammary membranes were frozen (-20°C). Other membrane preparations (porcine and bovine liver microsomes and porcine mammary microsomes) were frozen (-20°C) immediately after isolation until insulin binding assays could be performed.

^{125}I-insulin (specific activity = 80-130 µCi/µg) was prepared according to Freychet et al. (1971). Hormone integrity was determined by ^{125}I-insulin precipitability with trichloroacetic acid (TCA, 10%) and GPAIS. Only ^{125}I-insulin > 95% TCA precipitable and > 93% immunoprecipitable was employed in these studies.

The standard insulin binding assay consisted of 500 µg of membrane protein incubated with 0.6 ng (0.1 pmol) ^{125}I-insulin in 50 mM Tris/HCl, pH 7.8 at 4°C, containing 0.1% BSA. Final assay volume was 1 ml. For each determination, a parallel incubation was performed in the presence of excess unlabeled insulin (50 µg or 8.3 nmol). All incubations were in 12 mm x 75 mm polystyrene tubes, in triplicate, at 4°C for 36 - 48 h, with intermittent handmixing every 8 h. The binding reaction was terminated by the addition of 2 ml ice cold 0.1% BSA, Tris/HCl buffer followed by centrifugation at 5860 g for 30 min at 4°C. The supernatant was decanted, the tubes were drained, and the tube interiors were blotted above the membrane pellets with absorbent paper. Radioactivity in the pellets was counted for 10 min in a Nuclear Chicago automatic gamma counter (Series 1185) at an average counting efficiency of 75%. Specific ^{125}I-insulin binding to the membranes was determined by subtracting the number of counts bound to the pellet in the presence of excess unlabeled insulin (nonspecific binding) from the number of counts bound in the absence of excess unlabeled insulin (total binding). Specific binding was calculated as a percentage of the total radioactivity added to each incubation.

Least squares analyses of variance (Harvey, 1960) using a 2 x 2 factorial design was employed for analysis of the data in both studies. In Study 1, the effects of frozen storage (fresh vs freeze-thawed membranes) and mammary membrane source (milk vs mammary tissue) on insulin binding were tested. In Study 2, species (pig vs cow) and tissue (liver vs mammary) differences in insulin binding were determined.

RESULTS

Fresh bovine membranes (microsomes and MFGM) specifically bound similar amounts of ^{125}I-insulin as freeze-thawed membranes (microsomes and MFGM), and microsomes (fresh and freeze-thawed) specifically bound similar amounts of ^{125}I-insulin as MFGM (fresh and freeze-thawed) (Table 2). However, fresh mammary microsomes bound less insulin than did frozen mammary microsomes, fresh MFGM, or frozen MFGM ($P<0.09$) (Table 2).

Porcine microsomes bound more ($P<0.001$) insulin than did bovine microsomes from the same tissue (Table 3). Liver microsomes, regardless of species, bound more ($P<0.001$) insulin than did mammary microsomes (Table 3). Although only one porcine hepatic microsomal preparation

Table 2. Least squares means of insulin binding by fresh and freeze-thawed bovine mammary microsomes and milk fat globule membranes (MFGM).#,##

Membranes	n	Preparation (% Specific Binding) Fresh	Freeze-Thawed	S.E.M.
Microsomes	6	0.52	2.04	0.33
MFGM	6	1.76	1.63	0.33
S.E.M.		0.33	0.33	

Incubation duration = 36 h except for frozen microsomes, incubation duration = 48 h.
##Membrane x preparation interaction; P<0.09.

Table 3. Effect of species and tissue type on insulin binding by freeze-thawed microsomes.++

	Species## (% Specific Binding)		
Tissue+	Cow	Pig	S.E.M.
Mammary	2.04 (6)#	13.54 (3)	0.96
Liver	17.59 (6)	37.60 (1)	1.08
S.E.M.	0.83	1.44	

Least square means (n).
##Species differences, P<0.001.
+ Tissue differences, P<0.001.
++Species x tissue interaction, P<0.05.

was examined in this study, the percent specific binding reported is similar to that reported by Meserole and Etherton (1984) for liver microsomes from lean pigs (18-33% specific binding).

DISCUSSION

Membrane manipulation is known to affect polypeptide hormone binding (Posner et al., 1981). Posner et al. (1981) demonstrated that freeze-thawing enhanced insulin binding by isolated Golgi membranes. Since the isolated Golgi fraction exists predominantly in vesicular form (Ehrenreich et al., 1978), freeze-thawing enhanced insulin binding by disrupting the vesicles to expose receptors normally occluded inside. Mammary plasma membranes also form vesicles upon isolation, but MFGM isolated from bovine milk exist as small membranous sheets with free edges (Keenan et al., 1970).

Since microsomes are composed of rough and smooth endoplasmic reticulum, Golgi apparatus, and plasma membranes (Ehrenreich et al., 1978), it is not surprising that freeze-thawing of mammary microsomal preparations resulted in enhanced insulin binding. The nonvesicular nature of fresh MFGM would account for the higher insulin binding

observed in this preparation in comparison to fresh mammary microsomes. Likewise, freeze-thawing would not be expected to enhance insulin binding to MFGM since they are nonvesicular.

^{125}I-insulin binding by bovine MFGM was comparable to that demonstrated by freeze-thawed mammary microsomes. Further, frozen storage of MFGM (ave = 37 days) had little effect on binding. Since MFGM are of plasma membrane origin and binding by MFGM is comparable to binding by mammary microsomes, insulin binding to this fraction may reflect insulin binding by mammary epithelial cells.

This model is advantageous in that milk, the membrane source, is readily available and that multiple sampling of animals is accomplished easily. Examination of insulin binding by MFGM of numerous animals in various physiological states would reveal the potential of this model for screening insulin receptor status in the mammary gland of the dairy cow.

Specific insulin binding is generally assumed to reflect insulin receptor numbers. From this, bovine tissues appear to possess fewer insulin receptors than porcine tissues. Cattle also have lower plasma insulin concentrations than do swine (Horino et al., 1968). Differences in plasma insulin concentrations and insulin receptor numbers

between these two species suggest fundamental differences in insulin regulation and responsiveness.

Insulin binding has been quantitatively associated with glucose metabolism (glycogenesis, glycolysis, and 2-deoxyglucose uptake) in murine muscle (LeMarchand et al., 1977) and amino acid transport in rat hepatocytes (Dolais-Kitabgi et al., 1981). Therefore, the higher insulin binding by liver microsomes observed in this experiment suggests that liver is more responsive to insulin than is mammary tissue. In view of the observed insensitivity of hepatic glucose metabolism in the ruminant to insulin (Brockman, 1978), high insulin binding by bovine liver microsomes seems paradoxical. However, cellular responsiveness does not appear to be dictated by the level of insulin binding alone.

Cells possess spare insulin receptors (Olefsky & Ciaraldi, 1981). The "spare receptor" theory of hormone action maintains that a cell possesses more receptors than are required to elicit the maximal cellular response (Olefsky & Ciaraldi, 1981). When cellular insulin receptor occupancy reaches 10% or greater, the metabolic response is often maximal (Olefsky, 1975). Increased insulin binding results in increased cellular responsiveness only up to a critical level of receptor occupancy. After

the critical level of occupancy is reached, no further increase in cellular responsiveness is observed since postreceptor events become the rate limiting steps in insulin action (Olefsky & Ciaraldi, 1981). Our previous studies (Smith et al., 1986) indicated that occupancy of both bovine hepatic and mammary insulin receptors (K_d = 7.6×10^{-10}M and 9.6×10^{-11}M, respectively) should exceed 10% at physiological concentrations of plasma insulin (1.4×10^{-10}M) in the dairy cow. Hence, the "spare receptor" theory would predict maximal stimulation of bovine liver and mammary tissue by normal plasma insulin levels in ordinary physiological circumstances.

In addition, structural arrangement of insulin receptors in the plasma membrane may affect tissue responsiveness. Rat hepatocytes possess approximately five times more insulin receptors than do rat adipocytes (Olefsky & Ciaraldi, 1981); yet, rat liver is less sensitive to insulin than rat adipose tissue (Jarett et al., 1980). Jarett et al. (1980) reported that insulin receptors on rat adipocytes exist primarily in groups of two or more while insulin receptors on rat hepatocytes are primarily solitary. They proposed that the presence of receptor groups on rat adipocytes could increase the probability of receptor cross-linking, which is thought to intensify insulin

action, and thereby increase the sensitivity of adipocytes to insulin (Jarett et al., 1980).

Finally, it should be noted that insulin binding to the total microsomal fraction does not necessarily reflect insulin binding to the plasma membrane of the cell. Krupp and Lane (1981) found that chronic exposure of chick liver cells to insulin caused down-regulation of the number of insulin receptors in the plasma membrane, but had no effect on the total number of cellular receptors. However, Kelly et al. (1974) did detect significant differences in insulin binding by liver microsomes from rats and guinea pigs in various physiological states. While the latter study suggests that alterations in cellular insulin binding can be detected using microsomes, the results of Krupp and Lane (1981) suggest that caution must be exercised in making inferences about insulin binding to the plasma membrane solely on the basis of insulin binding data generated using the microsomal model.

At the time this work was conducted, insulin receptors had been identified in bovine liver plasma membranes (Rosen et al., 1979); however, information concerning insulin receptors in other bovine membranes was lacking.

Since then, insulin receptors have been identified in bovine liver microsomes (Smith et al., 1986), mammary microsomes (Oscar et al., 1986; Smith et al., 1986), and mammary smooth membranes (Oscar et al., 1986). Insulin receptors have also been described in bovine adipocytes (Vernon et al., 1985).

While these studies have provided direct measurements of insulin receptors in bovine tissues, they have not allowed conclusions about tissue responsiveness to insulin. However, insulin binding data, whether in membranes or cells, is important for understanding insulin action in bovine tissues. Further work concerning postreceptor mechanisms of insulin action, structural arrangement of receptors in the plasma membrane, cellular distribution of receptors, and metabolic response in relation to insulin binding will be needed before insulin binding data can be accurately interpreted in terms of tissue responsiveness.

LIST OF REFERENCES

Bergman, E. M., Brockman, R. P. & Kaufman, C. F. (1974) Fed. Proc. 33, 1849-1854

Brockman, R. P. (1978) Can. Vet. J. 19, 55-62

Dolais-Kitabgi, J., Checler, F., Rey, J., Morin, O. & Freychet, P. (1981) FEBS Letters 128, 321-324

Dowben, R. M., Brunner, J. R. & Philpott, D. E. (1967) Biochim. Biophys. Acta 135, 1-10

Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, C. P. & Palade, G. E. (1978) J. Cell. Biol. 59, 45-72

Flint, D. J. & West, D. W. (1983) Br. Vet. J. 139, 39-40

Freychet, P., Roth, J. & Neville, D. M., Jr. (1971) Biochem. Biophys. Res. Comm. 43, 400-408

Ganong, W. F. (1975) Review of Medical Physiology, 7th edn., p. 253, Lange Medical Publications, Los Altos

Harvey, W. R. (1960) USDA Res. Ser. ARS-20-80

Horino, M., Machlin, L. J., Hertelendy, F. & Kipnis, D. M. (1968) Endocrinology 83, 118-128

Jarett, L., Schweitzer, J. B. & Smith, R. M. (1980) Science 210, 1127-1128

Jarrett, I. G., Filsell, O. H. & Ballard, F. J. (1974) Horm. Metab. Res. Suppl. Series 4, 111-116

Kahn, C. R. (1975) in Methods in Membrane Biology, (Korn, E. D., ed.), vol. 3, pp. 81-146, Plenum Press, New York

Keenan, T. W., Morre, D. J., Olson, D. E., Yunghans, W. N. & Patton, S. (1970) J. Cell. Biol. 44, 80-93

Kelly, P. A., Posner, B. I., Tsushima, T. & Friesen, H. G. (1974) Endocrinology 95, 532-539

Krupp, M. & Lane, M. D. (1981) J. Biol. Chem. 256, 1689-1694

LeMarchand, Y., Loten, E. G., Assimacopoulos-Jeannet, F., Forgue, M., Freychet, P. & Jeanrenaud, B. (1977) Diabetes 26, 582-590

Lindsay, D. B. (1978) Biochem. Soc. Trans. 6, 1152-1156

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. 193, 265-275

Mather, I. H. & Keenan, T. W. (1983) in Biochemistry of Lactation, (Mepham, T. B., ed.), pp. 231-283, Elsevier Science Publishers B. V., Amsterdam

Meserole, V. K. & Etherton, T. D. (1984) J. Animal Sci. 59, 650-657

Olefsky, J. M. (1975) J. Clin. Invest. 56, 1499-1508

Olefsky, J. M. & Ciaraldi, T. P. (1981) in Diabetes Mellitus, (Brownlee, M., ed.), vol. 2, pp. 73-116, Garland STPM Press, New York

Oscar, T. P., Baumrucker, C. R. & Etherton, T. D. (1986) J. Animal Sci. 62, 179-186

Posner, B. I., Bergeron, J. J. M., Josefsberg, Z., Kahn, M. N., Khan, R. J., Patel, B. A., Sikstrom, R. A. & Verma, A. K. (1981) Rec. Prog. Horm. Res. 37, 539-582

Posner, B. I., Kelly, B. A., Shiu, R. P. C. & Friesen, H. G. (1974) Endocrinology 95, 521-531

Rosen, P., Ehrich, B., Junger, E., Bubenzer, H. J. & Kuhn, L. (1979) Biochim. Biophys. Acta 587, 593-605

Smith, D. H., Palmquist, D. L. & Schanbacher, F. L. (1986) Comp. Biochem. Physiol., in the press

Vernon, R. G., Finley, E., Taylor, E. & Flint, D. J. (1985) Endocrinology 116, 1195-1199

Wiman, K., Curman, B., Tragardh, L. & Peterson, P. A. (1979) Eur. J. Immunol. 9, 190-195

Yalow, R. S. & Berson, S. A. (1960) J. Clin. Invest. 39, 1157-1175

CHAPTER IV

Effect of Dietary Fat and Lactation Status on Insulin Binding to Bovine Milk Fat Globule Membranes

INTRODUCTION

The degree to which a cell will respond to a hormone depends on its effective hormone receptor concentration. Effective hormone receptor concentration may be defined as the number of hormone receptors per cell capable of interacting with the hormone to elicit an intracellular response. This concentration is a function of both the number of cell surface receptors available for binding and the binding affinity of these receptors for their hormone (25).

Effective hormone receptor concentration of a cell is not constant but fluctuates in response to physiological changes occurring within the organism. In the case of the insulin receptor, one of the primary physiological factors affecting receptor regulation in the normal cell is plasma insulin concentration (1). Hyperinsulinemia induces down

regulation of the effective receptor concentration, protecting the cell from overstimulation by insulin. Conversely, hypoinsulinemia induces up regulation of insulin receptors, preventing understimulation (1).

Many physiological and environmental factors, such as circulating hormone concentrations (extrainsulin) (12, 20), degree of physical training (16), disease (1), obesity (23), energy intake (24), and dietary carbohydrate intake (27), affect insulin receptor regulation by altering plasma insulin concentration. Growth status (26), age (28), and dietary fat intake (25) have been shown to regulate insulin receptors independently of plasma insulin concentration. High dietary fat intake has been implicated in defective synthesis of liver plasma membrane glycoproteins (7) by depressing the activities of hepatic galactosyl and sialyltransferases (37). Since the insulin receptor is a glycoprotein (10), dietary fat intake may result in defective insulin receptor synthesis by the cell, and hence, decreased insulin binding.

Insulin receptors have been characterized in bovine mammary microsomes (34). However, insulin binding to mammary microsomal preparations from lactating animals was highly variable (coefficient of variation = 92%, n = 6). Examination of the relationship between insulin binding to

bovine mammary tissue and lactation status (stage of lactation and milk yield) could provide information regarding insulin's role in metabolism of the ruminant mammary gland. Unfortunately, a large amount of mammary tissue (3-10 g) is required to prepare a sufficient quantity of microsomes for insulin receptor analysis (unpublished observation). Since the required amount of tissue can be obtained only by slaughter, which is costly, or massive mammary gland biopsy, which jeopardizes subsequent mammary health and function (18), survey investigation of insulin binding to mammary microsomes is unfeasible. Furthermore, neither method readily permits repeat sampling of individual animals.

An alternate source of mammary cell membranes is milk. When milk fat droplets are secreted from the mammary epithelial cell into the alveolar lumen, they are encased in a portion of the cell's apical plasma membrane (19). These membranes, referred to as milk fat globule membranes (MFGM), can be easily isolated from milk in large quantities (2). Flint and West (3) reported the presence of an insulin receptor in MFGM isolated from murine and caprine milk. Since MFGM are derived from the apical plasma membrane of the mammary cell, fluctuations

in insulin binding to these membranes may reflect fluctuations in insulin binding to the mammary epithelial cell.

We initiated a study of insulin binding to bovine MFGM in order to examine the relationship between insulin binding and lactation status of the cow and to determine if insulin binding to MFGM is modulated by dietary parameters, particularly dietary fat intake.

MATERIALS AND METHODS

Experimental Animals. Two studies were performed. In Study 1, 16 lactating cows were randomly chosen from the OARDC milking herd to evaluate the relationship between insulin binding to MFGM and lactation status (stage of lactation and level of milk production). In Study 2, 14 lactating cows, which were concurrently part of a study to evaluate the effect of dietary fat on milk and milk fat production, were chosen to evaluate the effect of dietary fat on insulin binding to MFGM.

In the dietary study, each treatment group received equal amounts of alfalfa hay and a standard concentrate mix composed of ground corn, supplemental protein as soybean meal and dried distillers grains, and supplemental vitamins and minerals balanced to meet National Research

Council requirements (21). Added dietary fat (Unifat M-37) took the place of corn in the concentrate mix. The treatments were as follows: Ø (N=8), 6 (n=2), 8 (n=2), and 12 (n=2) percent fat in the concentrate mix. Initially, two animals were allotted to each treatment group; however, six control animals later became available and were included in the analysis to increase sample size. All milk samples were taken after a dietary adjustment period of 2 wk.

Laboratory Procedures. Morning milk samples were obtained from all cows. The MFGM were prepared from the fresh, uncooled milk samples using 250 mM sucrose, 2 mM $MgCl_2$, pH 7.5 according to Dowben et al. (2) except that all initial wash steps were executed at 20°C instead of 35°C. This modification was to hinder insulin receptor degradation which is accelerated at higher temperatures (25). Freshly isolated MFGM were diluted in iced 50 mM Tris (hydroxymethyl) aminomethane (Tris-HCl), 10 mM $CaCl_2$ buffer, pH 7.5 at 4°C and membrane protein content measured according to Lowry et al. (17) using bovine serum albumin (BSA; Miles Biochemical, Elkhart, IN) as a standard. All binding studies were done using fresh MFGM preparations.

For insulin binding studies, [^{125}I] insulin (specific activity = 120.2 $\pm$ 22.8 μCi/μg) was synthesized with carrier-free Na ^{125}I in 0.1 NaOH (350 - 600 mCi/ml) (New England Nuclear, Boston, MA) and bovine crystalline insulin (26.6 U/mg) (courtesy of W. Fields, Eli Lilly Co.) using chloramine T (Sigma Chemical Co., St. Louis, MO) (4) as described elsewhere (34). The radiolabeled preparation was regarded suitable for binding studies if $\geq$ 95% of the tracer could be precipitated with 10% TCA and $\geq$ 93% of the tracer could be immunoprecipitated using guinea pig antibovine insulin serum (Miles Biochemical, Elkhart, IN).

To determine total [^{125}I] insulin binding, MFGM (500 μg Lowry protein) were added to .6 ng (.1 pmole) [^{125}I] insulin in 50 mM Tris-HCl, .1% BSA, 1 mM bacitracin (Sigma Chemical Co., St. Louis, MO), pH 8.0 at 20°C in a final volume of 1 ml. Nonspecific insulin binding was measured in parallel incubations containing an additional 50 μg (8.3 nmole) unlabeled insulin. All incubations were conducted in 12 x 75 mm polypropylene test tubes in quintuplicate at 20°C for 4.5 h. Incubations were hand agitated at the start of the assay to ensure proper mixing. The time and temperature conditions employed were previously determined to be optimal for the establishment of

equilibrium binding to the insulin receptor of bovine mammary microsomes (34).

Incubations were terminated by adding 2 ml ice-cold Tris-HCl, BSA buffer to each tube. Bound vs free radioactivity were separated by centrifugation at 19,600 x g for 30 min at 4°C. The resulting supernatants were decanted, the tubes drained, and the inside walls of each tube above the MFGM pellet blotted with absorbent paper. Pellets were counted for 10 min in a gamma counter (Nuclear Chicago 1185 Series, Automatic Gamma Counting System) operating at an average efficiency of 75% to assess bound, radiolabeled insulin. Total binding refers to the radioactivity (counts) bound to the MFGM pellet in the absence of excess, unlabeled insulin. Nonspecific binding refers to the radioactivity bound to the pellet in the presence of excess, unlabeled insulin, i.e, nonreceptor binding. Specific binding, i.e., receptor binding, was obtained by subtracting nonspecific binding from total binding and is expressed here as a percentage of the total radioactivity added to each incubation.

Statistical Analysis. In Study 1, percent specific insulin binding was regressed in a stepwise manner (22) on the following variables: daily milk yield (kg), stage of

lactation (d), breed (Holstein vs Jersey), age (yr), lactation number, daily milk fat yield (kg), milk fat percent, daily milk protein yield (kg), milk protein percent, breeding status (pregnant vs open), body weight (kg), metabolic body size (body weight$^{\cdot 75}$), and mammary health (mastitic vs nonmastitic).

In this analysis, none of the independent variables were responsible for the variation in percent specific insulin binding. Only daily milk yield had a marginal effect (r^2 = .13, P = .18). Since the 16 animals in Study 1 were randomly selected from the milking herd, a wide range in milk production among these animals was not insured. Individual milk yields for these cows only ranged from 3.4 to 28.2 kg/d (14.3 $\pm$ 6.9 kg/d, mean $\pm$ SE). A lack of high producing cows in our sample could have accounted for the failure to detect a relationship between milk production and insulin binding. Individual milk yields for control cows (no added dietary fat) in Study 2 ranged from 13.0 to 41.1 kg/d with a mean daily production of 31.9 $\pm$ 9.0 kg/d. In order to expand the milk production range as well as to increase sample size, the binding data from Study 1 was pooled with the binding data of the control cows of Study 2 and the regression analyses repeated.

In Study 2, the effect of dietary fat on percent specific insulin binding, daily milk yield, daily milk fat yield, and daily milk protein yield was analyzed using the least squares analysis of variance (6). When treatment effect was significant ($P<0.1$), Duncan's multiple range test (35) was used to separate the means.

RESULTS

Table 4 lists simple correlations between percent specific insulin binding to MFGM and various physiological factors for the pooled specific binding data of cows in Study 1 and Study 2. The only variable correlated with percent specific insulin binding was daily milk yield [$r^2 = .20$, $Sy{\cdot}x$ (standard error of the estimate) = .81, $P<.05$]. The multiple regression analysis yielded the following prediction equation:

$$Y = .880 + .034\ X,$$

where Y = percent specific insulin binding to MFGM and X = daily milk yield (kg). Although the level of daily milk production influenced insulin binding to MFGM, it accounted for only 20% of the variability in binding. Therefore, milk production alone is not adequate to predict accurately percent specific insulin binding to MFGM.

Table 4. Simple correlation between percent specific insulin binding to milk fat globule membranes (MFGM) and physiological variables.

Variable	Correlation Coefficient (r)
Stage of Lactation	.24
Breed	-.11
Age of Cow	-.09
Lactation Number	-.07
Daily Milk Yield	.44*
Daily Milk Fat Yield	.16
Milk Fat Percentage	-.07
Daily Milk Protein Yield	.38
Milk Protein Percentage	-.08
Body Weight	.04
Mammary Health (Mastitis)	-.15
Breeding Status (Pregnant vs Open)	.21
Metabolic Body Size	.05

*P<.05

It should be noted that the effect of daily milk production on percent specific insulin binding was significant ($P<.05$) only when the control animals of Study 2 were included in the analysis. Failure to detect a relationship between milk production and specific binding in Study 1 probably resulted from the limited range in milk production exhibited by these cows. Inclusion of the control cows of Study 2 in the analysis broadened the milk production range so that a relationship could be detected.

The effects of dietary fat on insulin binding and production parameters are summarized in Table 5. Dietary fat had a significant ($P<.06$) effect on specific insulin binding to MFGM, but had no effect on any of the production variables. Specific insulin binding increased with increasing dietary fat up to a level of 8% additional fat in the concentrate mix. Thereafter (12% fat in the concentrate mix), additional fat had no additional effect on specific binding.

Linear regression of percent additional fat in the concentrate mix on insulin binding to MFGM (Figure 12) yielded the following equation:

$$Y = 2.06 + .21\ X\ (r^2 = .47,\ P<.01)$$

Table 5. The effect of additional dietary fat on specific insulin binding to MFGM, daily milk yield, daily milk fat yield, and daily protein yield.*

Item	Dietary Fat Level Added To The Concentrate (%) 0	6	8	12	SEM**
Number of Cows	8	2	2	2	--
Specific Insulin Binding to MFGM (%)	2.02[a]	3.21[ab]	4.38[b]	4.19[b]	.37
Daily Milk Yield (kg)	32.7	29.9	34.2	21.0	2.38# (NS)#
Daily Fat Yield (kg)	.65	.85	.42	.43	.09 (NS)
Daily Protein Yield (kg)	.94	.86	1.05	.63	.07 (NS)

* Least square means.
**Standard error of the mean.
Nonsignificant.
[ab]Means in the same row with different superscripts differ (P<.06).

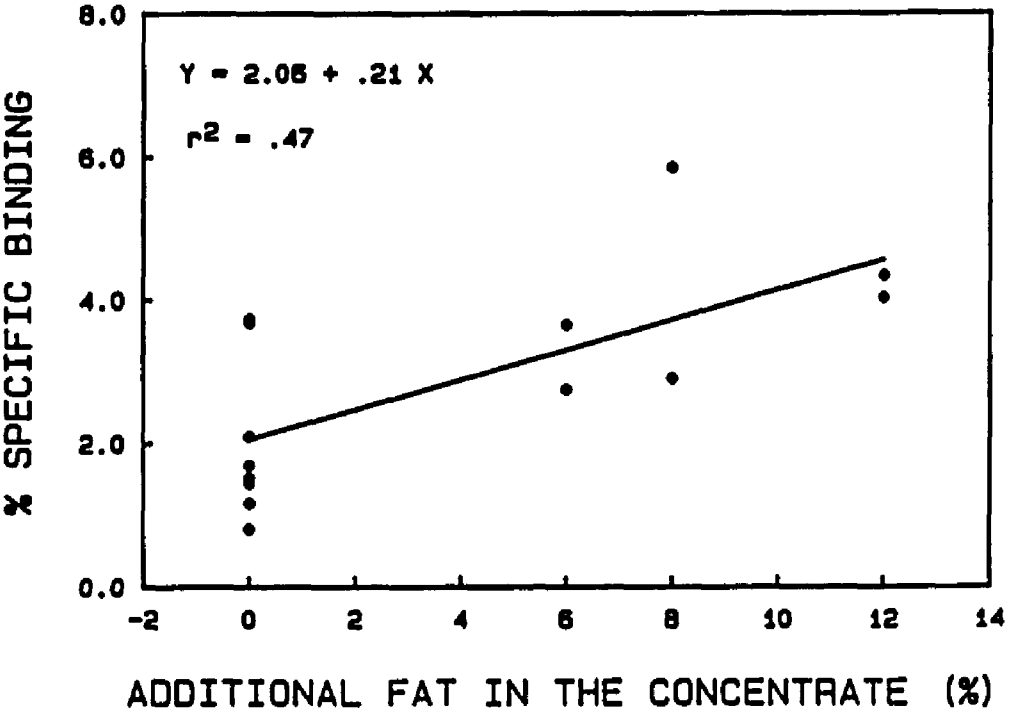

Figure 12. Relationship between percent additional fat in the concentrate mix and percent specific ^{125}I-insulin binding to MFGM.

where Y = percent specific insulin binding to MFGM and X = percent additional fat in the concentrate mix. The intercept of 2.06 closely corresponds to the mean percent specific binding for the control cows of Study 2 (2.02 $\pm$ .41%) confirming that animals receiving a conventional diet (ca 3% fat) have a baseline level of approximately 2.0% specific insulin binding to their MFGM. The relationship between fatty acids ingested and percent specific insulin binding (Figure 13) was also significant (r^2 = .41, $P<.01$).

DISCUSSION

The origin of the insulin receptors in the MFGM is unknown, but they presumably originate from the apical plasma membrane of the mammary secretory cell along with the MFGM itself. From existing knowledge of the dynamics of cellular receptors (32), three possible routes can be postulated for insulin receptor deposition in the apical membrane: 1) intramembrane receptor diffusion from the basolateral membranes; 2) intracellular receptor biosynthesis and apical membrane insertion; and 3) intracellular translocation of receptors from the basolateral membranes.

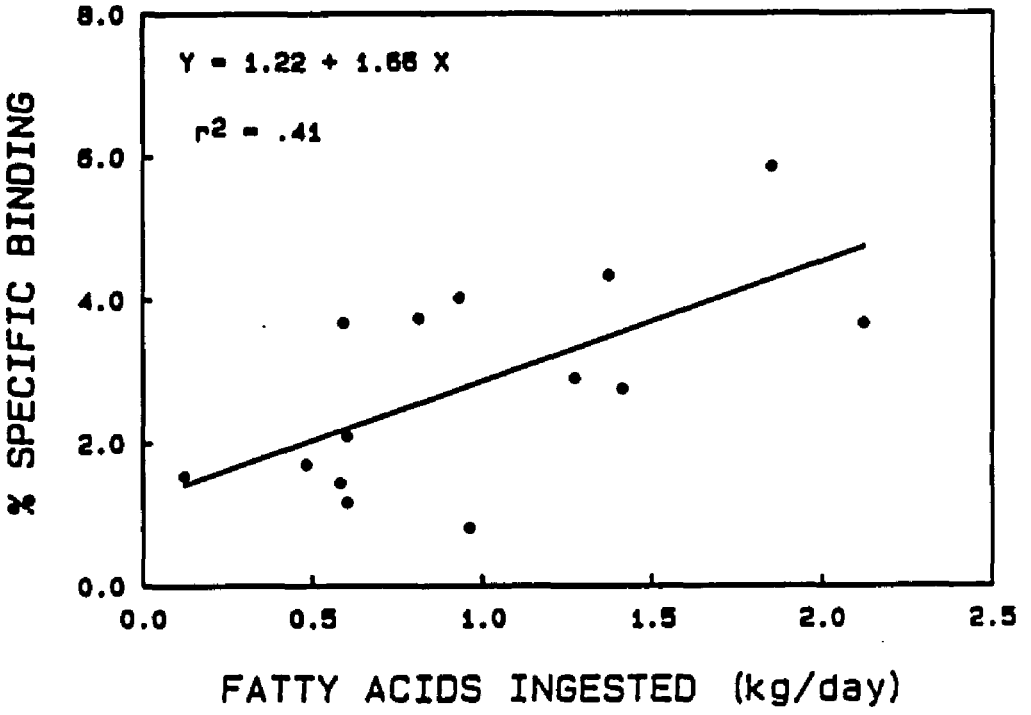

Figure 13. Relationship between kg fatty acids ingested and percent specific ^{125}I-insulin binding to MFGM.

Intramembrane receptor diffusion from the basolateral to the apical surface is improbable due to the intervening tight junctions at the apical border of mammary epithelial cells (31). These tight junctions join neighboring cells and prevent basoapical intramembrane diffusion of membrane components.

Receptor biosynthesis and specific membrane insertion is possible but would imply a functional role for the insulin receptor at the apical surface of the mammary cell. Insulin has been found in milk (14); therefore, the apical surface of the mammary cell, and presumably any receptors thereon, is exposed to insulin. A regulatory role for milk insulin has not been established, but insulin could potentially trigger intracellular events via the apical insulin receptor. Since the tight junctions should prevent paracellular transport of insulin into milk (31), insulin appears to be secreted directly into the milk by the epithelial cell. Therefore, it is difficult to imagine how milk insulin could be an effective regulator of mammary cell function via insulin receptors on the apical cell surface.

The third possible source of apical insulin receptors is intracellular translocation of receptors from the basolateral membrane. In general, the insulin-receptor

complex is internalized within 5 min of hormone binding (32). Complexes usually localize at the lysosomes where both insulin and the receptor may be degraded. However, receptor recycling to the plasma membrane can occur. Recently, King and Johnson (13) provided in vitro evidence that intact insulin-receptor complexes are rapidly internalized from the lumenal plasma membrane of bovine endothelial cells and unidirectionally translocated to the extravascular membrane. Their work implicates the insulin receptor in insulin transport from the blood to the tissue space.

The mammary epithelial cell, like the endothelial cell, is polarized and displays unidirectional transport of substrates from the basal to apical surface (31). It is possible that mammary epithelial cells also display unidirectional translocation of insulin receptors from their basolateral surface to the apical membrane. Such translocation could account for the insulin receptor in MFGM as well as insulin in milk. Secretion of insulin-receptor complexes into the alveolar lumen by the cell might represent a simple alternative to lysosomal degradation of the complex.

In Study 1, daily milk yield was found to be positively associated with specific insulin binding to MFGM.

Hart et al. (5) have demonstrated that plasma insulin concentration is inversely related to milk production in lactating cattle. Since plasma insulin concentration is also inversely related to effective insulin receptor concentration (25), high insulin binding to the MFGM of high producing dairy cattle may reflect up regulation of insulin receptors in the mammary gland in response to hypoinsulinemia.

Jones et al. (11) examined insulin metabolism in lactating and nonlactating rats. They reported that during lactation, [^{125}I] insulin uptake by the gland was increased 12-fold and the metabolic clearance rate of insulin was doubled. Furthermore, [^{125}I] insulin uptake by the gland was inhibited in the presence of unlabeled insulin indicating that the uptake process was receptor mediated. They suggested that lactation triggers a state of increased insulin sensitivity in the mammary gland resulting in increased insulin uptake and degradation via specific insulin receptors.

It is possible that the more metabolically active mammary gland of the high producing dairy cow extracts more insulin that that of the low producing cow. Although internalization of the insulin-receptor complex is usually followed by lysosomal degradation, the mammary cell, as

the endothelial cell, may bypass this step and release insulin-receptor complexes into the milk via the MFGM. In this case, MFGM isolated from high producing cows would be expected to bind more insulin. Unfortunately, insulin-receptor complex internalization and lysosomal degradation have never been examined in the mammary cell; therefore, the fate of the complex beyond the plasma membrane is unknown. However, the presence of insulin in milk suggests that insulin is internalized via the insulin receptor.

In Study 2, ingestion of dietary fat in excess of the baseline level provided by the conventional lactation diet (ca 3% fat) resulted in increased insulin binding to MFGM. Although increased milk production and milk fat production have been reported in dairy cattle fed high fat diets (29), neither milk nor fat production was altered by dietary fat in this study (Table 5). These findings suggest that the observed increase in insulin binding to MFGM was not the result of a diet-induced increase in milk fat secretion.

Insulin resistance has been associated with the ingestion of high levels of fat in nonruminants. This resistance is characterized by impaired cellular glucose transport (8, 15), glucose metabolism (9, 15), and insulin binding (9, 36). A reduction in the number of cellular

insulin receptors is responsible for the decreased insulin binding observed (9, 36) and has been implicated as a possible cause of this dietary-induced condition.

Palmquist and Moser (30) reported that feeding high fat diets to dairy cattle also induces insulin resistance as evidenced by impaired glucose clearance and enhanced insulin secretion in response to an i.v. glucose injection. If high dietary fat levels induce insulin resistance in ruminants and nonruminants by a similar mechanism, fat feeding of dairy cattle should depress insulin binding to bovine tissues as well. Our study suggests that fat feeding has the opposite effect in bovine mammary tissue, i.e., increased insulin binding. However, the validity of this conclusion hinges on the assumption that insulin binding to the MFGM corresponds to insulin binding to the mammary cell.

It should be noted that the nonruminant studies documenting a relationship between impaired insulin binding and fat feeding employed male rats (9, 36). Furthermore, these studies limited their examination of insulin binding to two tissues; adipose and liver. In a survey of 15 tissues from seven different animal species, Posner et al. (33) found considerable species and tissue differences in insulin binding. We have also observed significant

species (pig vs cow) and tissue (liver vs mammary) differences in insulin binding (unpublished observation). It is possible, in light of these differences, that the insulin binding response to high fat feeding differs as a function of species, tissue, or gender. Additional research is needed before any definitive conclusions concerning the relationship of fat feeding to insulin resistance can be drawn.

In conclusion, both level of milk production and level of dietary fat ingested influenced insulin binding to MFGM. These results suggest that physiological changes induce insulin receptor regulation in the bovine mammary gland. Studies characterizing the insulin receptor on the mammary cell proper vs the MFGM, as well as studies dealing with the origin of the insulin receptor on MFGM are needed before the significance of insulin receptor regulation on MFGM can be understood. Nonetheless, our results suggest that MFGM insulin receptor levels may be a useful and convenient study tool to bridge the gap between studies in isolated cells vs the whole animal.

LIST OF REFERENCES

1. Blei, A. T., D. C. Robbins, E. Drobny, G. Baumann, and A. H. Rubenstein. 1982. Insulin resistance and insulin receptors in hepatic cirrhosis. Gastroenterology 83:119.

2. Dowben, R. M., J. R. Brunner, and D. E. Philpott. 1967. Studies on milk fat globule membranes. Biochim. Biophys. Acta 135:1.

3. Flint, D. J. and D. W. West. 1983. Insulin receptors in milk. Br. Vet. J. 139:39.

4. Freychet, P., J. Roth, and D. M. Neville, Jr. 1971. Monoiodoinsulin: Demonstration of its biological activity and binding to fat cells and liver membranes. Biochem. Biophys. Res. Comm. 43:400.

5. Hart, I. C., J. A. Bines, S. V. Morant, and J. L. Ridley. 1978. Endocrine control of energy metabolism in the cow: Comparison of the levels of hormones (prolactin, growth hormone, insulin and thyroxine) and metabolites in the plasma of high- and low-yielding cattle at various stages of lactation. J. Endocr. 77:333.

6. Harvey, W. R. 1960. Least-squares analysis of data with unequal subclass numbers. USDA Res. Ser. ARS-20-80.

7. Henriquez, D. S., H. M. Tepperman, and J. Tepperman. 1979. Effects of high-glucose and high-fat diets on concanavalin A binding to rat liver plasma membranes and on the amount and pattern of their glycoprotein carbohydrates. J. Lipid Res. 20:624.

8. Ip, C., H. M. Tepperman, J. DeWitt, and J. Tepperman. 1976. The effect of diet fat on rat adipocyte glucose transport. Horm. Metab. Res. 9:218.

9. Ip, C., H. M. Tepperman, P. Holohan, and J. Tepperman. 1976. Insulin binding and insulin response of

adipocytes from rats adapted to fat feeding. J. Lipid Res. 17:588.

10. Jacobs, S., E. Hazum, and P. Cuatrecasas. 1980. Digestion of insulin receptors with proteolytic and glycosidic enzymes - Effects on purified and membrane-associated receptor subunits. Biochem. Biophys. Res. Commun. 94:1066.

11. Jones, R. G., V. Ilic, and D. H. Williamson. 1984. Physiological significance of altered insulin metabolism in the conscious rat during lactation. Biochem. J. 220:455.

12. Kahn, C. R., I. D. Goldfine, D. M. Neville, Jr., and P. DeMeyts. 1978. Alterations in insulin binding induced by changes in vivo in the levels of glucocorticoids and growth hormone. Endocrinology 103:1054.

13. King, G. L. and S. M. Johnson. 1985. Receptor-mediated transport of insulin across endothelial cells. Science 227:1583.

14. Kulski, J. K. and P. E. Hartman. 1983. Milk insulin, GH and TSH: Relationship to changes in milk lactose, glucose and protein during lactogenesis in women. Endoc. Exper. 17:317.

15. Lavau, M., S. K. Fried, C. Susini, and P. Freychet. 1979. Mechanisms of insulin resistance in adipocytes of rats fed a high-fat diet. J. Lipid Res. 20:8.

16. LeBlanc, J., A. Nadeau, M. Boulay, and S. Rousseau-Migneron. 1979. Effects of physical training and adiposity on glucose metabolism and ^{125}I-insulin binding. J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 46:235.

17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

18. Marx, G. D. and E. V. Carnolo. 1963. Method of biopsy of the mammary gland of cows and goats. J. Dairy Sci. 46:576.

19. Mather, I. H. and T. W. Keenan. 1983. Function of endomembranes and the cell surface in the secretion of organic milk constituents. Pages 232 - 283 in

Biochemistry of lactation. T. B. Mepham, ed. Elsevier Science Publishers, BV, New York, N.Y.

20. Muggeo, M., R. S. Bar, J. Roth, C. R. Kahn, and P. Gorden. 1979. The insulin resistance of acromegaly: Evidence for two alterations in the insulin receptor on circulating monocytes. 48:17.

21. National Research Council. 1978. Nutrient requirements of dairy cattle. Natl. Acad. Sci., Washington, D.C.

22. Neter, J. and W. Wasserman. 1974. Pages 382 - 386 in Applied linear statistical models. R. D. Irwin, Inc., Homewood, Ill.

23. Olefsky, J. M. 1976. Decreased insulin binding to adipocytes and circulating monocytes from obese subjects. J. Clin. Invest. 57:1165.

24. Olefsky, J. M. 1976. Effects of fasting on insulin binding, glucose transport, and glucose oxidation in isolated rat adipocytes. J. Clin. Invest. 58:1450.

25. Olefsky, J. M. and T. P. Ciaraldi. 1981. The insulin receptor: Basic characteristics and its role in insulin resistant states. Pages 73 - 116 in Diabetes mellitus. M. Brownlee, ed. Garland STPM Press, New York, N.Y.

26. Olefsky, J. M. and G. M. Reaven. 1975. Effects of age and obesity on insulin binding to isolated adipocytes. Endocrinology 96:1486.

27. Olefsky, J. M. and M. Saekow. 1978. The effects of dietary carbohydrate content on insulin binding and glucose metabolism by isolated rat adipocytes. Endocrinology 103:2252.

28. Pagano, G., M. Cassander, A. Diana, E. Pisu, C. Bozzo, F. Ferrero, and G. Lenti. 1981. Insulin resistance in the aged: The role of the peripheral insulin receptors. Metabolism 30:46.

29. Palmquist, D. L. and H. R. Conrad. 1978. High fat rations for dairy cows. Effects on feed intake, milk and fat production, and plasma metabolites. J. Dairy Sci. 61:890.

30. Palmquist, D. L. and E. A. Moser. 1981. Dietary fat effects on blood insulin, glucose utilization, and milk protein content of lactating cows. J. Dairy Sci. 64:1664.

31. Pitelka, D. R. and S. T. Hamamoto. 1983. Ultrastructure of the mammary secretory cell. Pages 29 - 70 in Biochemistry of lactation. T. B. Mepham, ed. Elsevier Science Publishers, BV, New York, N.Y.

32. Posner, B. I., J. J. M. Bergeron Z. Josefsberg, M. N. Khan, R. J. Kahn, B. A. Patel, R. A. Sikstrom, and A. K. Verma. 1981. Polypeptide hormones: Intracellular receptors and internalization. Rec. Prog. Horm. Res. 37:539.

33. Posner, B. I., P. A. Kelly, R. P. C. Shiu, and H. G. Friesen. 1974. Studies of insulin, growth hormone and prolactin binding: Tissue distribution, species variation and characterization. Endocrinology 95:521.

34. Smith, D. H., D. L. Palmquist, and F. L. Schanbacher. 1986. Characterization of insulin binding to bovine liver and mammary microsomes. Comp. Biochem. Physiol. In Press.

35. Steel, R. G. D. and J. H. Torrie. 1980. Pages 187 - 188 in Principles and procedures of statistics. McGraw-Hill, Inc., New York, N.Y.

36. Sun, J. V., H. M. Tepperman, and J. Tepperman. 1977. A comparison of insulin binding by liver plasma membranes of rats fed a high glucose diet or a high fat diet. J. Lipid Res. 18:533.

37. Tepperman, H. M., R. Silver, J. DeWitt, and J. Tepperman. 1981. Effects of high glucose and high lard diets on the activities of rat liver glycosyltransferases. J. Nutr. 111:1734.

CHAPTER V

Effect of Fat Feeding on Insulin Binding to Rat Mammary and Liver Microsomes

INTRODUCTION

In rats, a high fat diet reduces insulin binding to adipocytes[1] and liver plasma membranes[2] by decreasing the number of insulin receptors. The mechanism for this modulation of insulin receptors is unknown; however, dietary fat can modulate membrane fluidity[3] and thereby influence insulin binding.[4]

In dairy cattle, feeding a high fat diet depresses milk protein percentage[5-7] and yield[6,7], and also causes insulin resistance and increased insulin secretion.[8]

In view of the stimulatory role of insulin in protein synthesis,[9] it is possible that dietary fat inhibits milk protein synthesis by modulating mammary insulin receptors. We used rat mammary tissue as a model to examine the effect of dietary fat on insulin receptors. Liver tissue was studied for comparative purposes. The Scatchard plot[10] was employed to graphically express all hormone binding data.

The Scatchard equation describes binding of a hormone (H) to a homogeneous class of independent receptors (R) with an affinity constant (K_a) to form a hormone-receptor complex (HR) as follows:

$$\frac{[HR]}{[H]} = K_a([R_o] - [HR]) \quad [11]$$

In this relationship, the ratio of bound to free hormone is expressed as a function of bound hormone to yield a straight line with abscissal intercept equal to $[R_o]$, the concentration of total receptor sites, and the slope equal to $-K_a$.

Not all hormone binding data conform to Scatchard linearization. Nonlinear Scatchard plots have been reported for ACTH, oxytocin, glucagon, TRH, TSH, acetylcholine, catecholemines, and insulin binding data.[12] No simple, single slope (binding affinity) or abscissal intercept (receptor concentration) can be calculated for nonlinear Scatchard plots; however, these curves can be resolved into two or more linear components. Each linear component is believed to represent a separate class of binding sites differing from each other in binding capacity and binding affinity.[13] The curvilinear Scatchard plots of insulin binding data are usually resolved into two linear components representing a high affinity-low capacity site and a low affinity-high capacity site.[11]

Previous insulin binding studies in our laboratory employing both bovine[14] and murine (unpublished observation) liver and mammary microsomes yielded the typical nonlinear Scatchard plots. However, computer fitting of these curves was difficult when the binding data were within a limited range of insulin concentrations. To facilitate characterization of insulin receptors in this study, insulin binding was examined using a broad and detailed range of insulin concentrations.

Kahn[15] pointed out that curvilinear Scatchard plots can be interpreted erroneously if the binding data analyzed are limited in range or inclusiveness. Several investigators[16-18] have reported only one class of insulin receptors based on limited binding data. The results of this study indicate that when the binding data are comprehensive, a model with three rather than two sites more accurately describes the insulin binding data.

MATERIALS AND METHODS

Experimental Animals. Fourteen untimed pregnant (16-19 days gestation) Sprague-Dawley albino rats were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were housed individually in a temperature-

controlled (23 °C) room with a 12 hour light-dark cycle. All animals were fed a stock diet (Ralston Purina, St. Louis, MO) and water ad libitum prior to the experimental diets. At parturition (day 1 of lactation), each litter was normalized to 8 pups.

On day 7 of lactation (day 1 of dietary treatment), dams were randomly assigned to one of the following diets: the stock diet (control), a high fat-low carbohydrate diet (HF-LC), and a high carbohydrate-low fat diet (HC-LF). Both the HF-LC and HC-LF diets were prepared as described by Lauvau et al[19] except that corn starch was substituted for wheat starch. The composition of the experimental diets is shown in Table 6. Rats were maintained on these diets for 8 days.

On day 15 of lactation (day 8 of dietary treatment), all dams were sacrificed by cervical dislocation. Livers and mammary glands were removed, fast-frozen in a dry ice bath, and stored at -20 °C until microsomal preparation.

Preparation of Microsomes. Microsomes were prepared from the freeze-thawed tissues as described previously[14] and stored at -20 °C for subsequent insulin receptor analysis. Microsomal protein concentration was determined by

Table 6. Composition of Experimental Diets.[19]*

Ingredient	HC-LF	HF-LC
	(g/100g)	
Casein	20	29
Lard	3	44
Corn oil	1	1
Corn starch	68	14
Vitamin mixture**	2	3
Salt mixture#	4	6
Cellulose	2	3

* HC-LF = high carbohydrate-low fat diet; HF-LC = high fat-low carbohydrate diet.
**Vitamin diet fortification mixture, ICN Corp., Cleveland, Ohio.
Salt mixture XIV, ICN Corp.

the Lowry method[20] using bovine serum albumin (BSA) as a standard.

Iodination of Insulin. Porcine insulin (26.8 U/mg) (gift of Eli Lilly Co., Indianapolis, IN) was iodinated to a specific activity of 189 µCi/µg with carrier-free Na ^{125}I (350 - 600 mCi/mL) (New England Nuclear Products, Boston, MA) using chloramine-T.[21] Iodinated insulin was regarded suitable for binding studies if 95% or more of the total radioactivity was precipitated by 10% trichloroacetic acid (TCA) and 93% or more was precipitated by guinea pig antibovine insulin serum (GPAIS) (Miles Biochemical, Elkhart, IN).

Binding of Insulin to Liver and Mammary Microsomes. Microsomes (500 µg Lowry protein) were incubated in 1 mL of 50 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl), 0.1% BSA (Miles Biochemical, Elkhart, IN), 1 mM bacitracin (Sigma, St. Louis, MO) pH 7.8 at 20 °C with iodinated insulin (0.6 ng; 0.1 pmole) and increasing concentrations of unlabeled insulin (0 - 1000 ng; 0.0 - 0.17 nmole). All incubations were performed in triplicate in 12 x 75 mm polypropylene test tubes at 20 °C. After a 1 hour equilibration period, 2 mL iced Tris-BSA buffer was added to

each tube. Bound and free radioactivity were separated by centrifugation at 19,600 g for 30 minutes at 4 °C. Supernatants were decanted, the tubes drained, and the tube interiors above the pellets swabbed with absorbent paper. Bound radioactivity was counted for 10 minutes in a γ-counter (The Automatic Gamma Counting System, 1185 Series, Nuclear Chicago) at 75% efficiency. Specific insulin binding was calculated by subtraction of radioactivity bound to the pellet in the presence of 50 μg (8.3 nmole) unlabeled insulin (nonspecific binding) from total insulin binding at each insulin concentration. Specific binding was expressed as a percentage of the total radioactivity added to each tube.

Binding affinities and receptor numbers for insulin were determined for individual rat liver and mammary microsomal samples by fitting the following binding equations for two (equation 1) and three (equation 2) classes of binding sites, respectively, using the NONLIN program of Metzler et al.[22]:

$$IR = \frac{n_1K_1[I]}{1+K_1[I]} + \frac{n_2K_2[I]}{1+K_2[I]} \qquad [1]$$

and

$$IR = \frac{n_1K_1[I]}{1+K_1[I]} + \frac{n_2K_2[I]}{1+K_2[I]} + \frac{n_3K_3[I]}{1+K_3[I]}, \qquad [2]$$

where IR = the mass of insulin bound per ml incubation
[I] = the concentration of free insulin,
n_1 = the number of binding sites in the first class of sites,
K_1 = the association constant for the interaction of I with a receptor in the first class of sites,
n_2 = the number of binding sites in the second class of sites,
K_2 = the association constant for the interaction of I with a receptor in the second class of sites,
n_3 = the number of binding sites in the third class of sites,
K_3 = the association constant for the interaction of I with a receptor in the third class of sites.

Following analysis, data were converted to Scatchard[10] format for display.

Statistical Analysis. Results were analyzed statistically using least squares analysis of variance.[23] When statistical significance was found, mean differences were separated by Duncan's multiple range test.[24]

RESULTS AND DISCUSSION

Average Scatchard plots for insulin binding by rat liver and mammary microsomes are shown for each dietary treatment in Figures 14 and 15, respectively. Curves within each tissue were very similar despite different treatments. Table 7 shows that the affinity constants

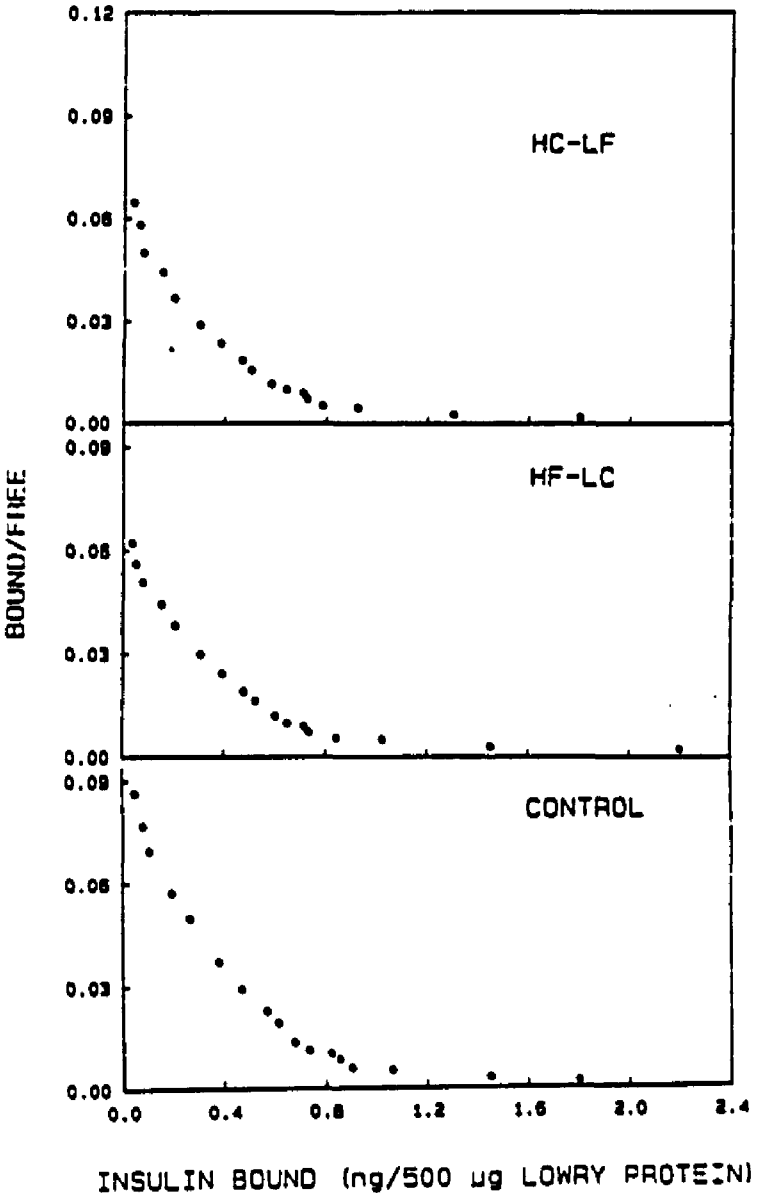

Figure 14. Scatchard plots for insulin binding to liver microsomes of rats fed high carbohydrate-low fat (HC-LF), high fat-low carbohydrate (HF-LC), or control diets. Upper panel, HC-LF; middle panel, HF-LC; and lower panel, control.

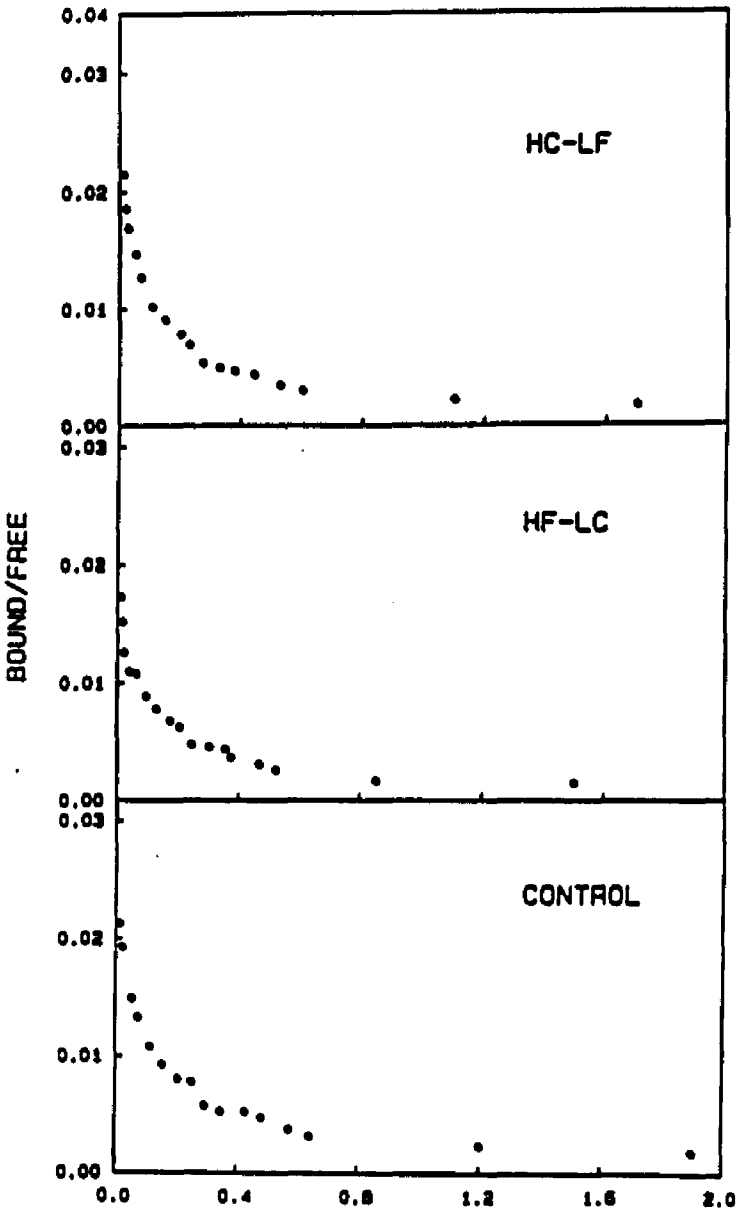

Figure 15. Scatchard plots for insulin binding to mammary microsomes of rats fed high carbohydrate-low fat (HC-LF), high fat-low carbohydrate (HF-LC), or control diets. Upper panel, HC-LF; middle panel, HF-LC; and lower panel, control.

Table 7. Effect of Diet on the Binding Capacities and Binding Affinities of Insulin in Rat Liver and Mammary Microsomes - Two Site Model.[§,+]

	Diet*			
Measurement**	Control	HF-LC	HC-LF	SEM++
Liver				
n	3	5	5	
K_{a1}	8.915 (8.2×10^{8})	8.802 (6.3×10^{8})	8.832 (6.8×10^{8})	0.036
K_{a2}	6.940 (8.7×10^{6})	6.516 (3.3×10^{6})	6.883 (7.6×10^{6})	0.134
R_1	1.0×10^{-13}	9.7×10^{-14}	9.4×10^{-14}	9.9×10^{-15}
R_2	3.4×10^{-13}	7.7×10^{-13}	4.0×10^{-13}	1.2×10^{-13}
Mammary				
n	2	5	5	
K_{a1}	8.989 (9.7×10^{8})	8.925 (8.4×10^{8})	8.733 (5.4×10^{8})	0.063
K_{a2}	7.109 (1.3×10^{7})	7.241 (1.7×10^{7})	6.839 (6.9×10^{6})	0.134
R_1	1.4×10^{-14}	1.7×10^{-14}	3.0×10^{-14}	3.6×10^{-15}
R_2	3.0×10^{-13}	3.1×10^{-13}	4.6×10^{-13}	7.1×10^{-14}

* HF-LC = high fat-low carbohydrate diet; HC-LF = high carbohydrate-low fat diet.

** n = number of animals, K_{a1} = binding affinity of the high affinity-low capacity site, K_{a2} = binding affinity of the low affinity-high capacity site, R_1 = binding capacity of the high affinity-low capacity site, R_2 = binding capacity of the low affinity-high capacity site.

§ Binding affinities are listed as log K_a and as $K_a(M^{-1})$, directly below in parentheses; Binding capacities are moles insulin bound/500 µg Lowry protein.

\+ Values are least square means.

++ SEM listed for K_a are in terms of log K_a.

(K_a) and binding capacities (R) estimated using the NONLIN program for two classes of receptor sites for both the liver and mammary microsomes were not influenced by diet ($P>0.10$). However, because computer fitting of the binding data using the two site model was difficult, we believe that these binding data are not valid.

For the majority of curves, several obvious outliers were apparent which prevented accurate fitting of the data. Elimination of these observations improved the fit; however, the deviations from the computed curves were usually nonrandom, indicating that the two site model was not appropriate for the data. Furthermore, the uncertainties of the binding parameter estimates (K_a and R values) were large (>100% error in each case) suggesting that the estimates were poorly defined. Three of the analyses (one liver and two mammary) were eliminated totally from the study due to an insufficient number of data points within a critical region of the curve after elimination of outlying points.

Visual examination of the individual Scatchard plots for both liver and mammary microsomes suggested that a model with three rather than two sites might provide a better fit of the binding data. In 16 of the 25 evaluable

Scatchard plots, definition of three sites was not visually apparent, but in the remaining nine plots (3 liver and 6 mammary), three binding sites were clearly defined. One of these nine Scatchard plots is shown in Figure 16. Consequently, all binding data were fit using the NONLIN program for three classes of binding sites and the results compared with those of the two site model. Selection of the appropriate model was based on the following criteria: 1) randomness of deviations from the computed curve; 2) confidence in the NONLIN parameter estimates as measured by estimate/standard deviation (t); 3) convergence of computer iterations in achieving a final fit of the data; and 4) the graphic display of the experimental data.

The three site model was superior to the two site model in fitting all 13 evaluable liver studies. The three site model was also superior in fitting nine of the twelve evaluable mammary studies. On the other hand, the two site model provided a better fit for the three remaining mammary analyses.

Figures 17 and 18 demonstrate the superiority of the three site model in fitting the liver and mammary binding data, respectively. When the three site model was used to fit the data, a high affinity receptor site was detectable (lower panels) which was not apparent when the two site

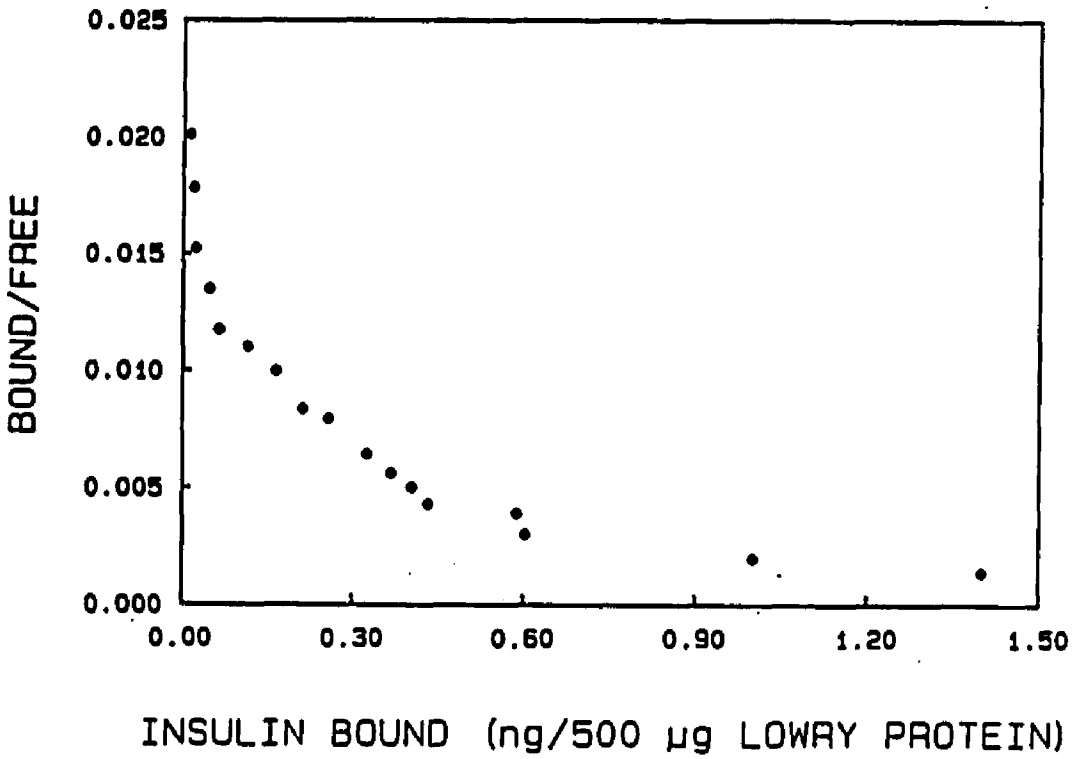

Figure 16. Scatchard plot of insulin binding to rat mammary microsomes exhibiting three classes of insulin receptors. This plot is representative of the remaining eight plots in which three classes of binding sites were visually apparent.

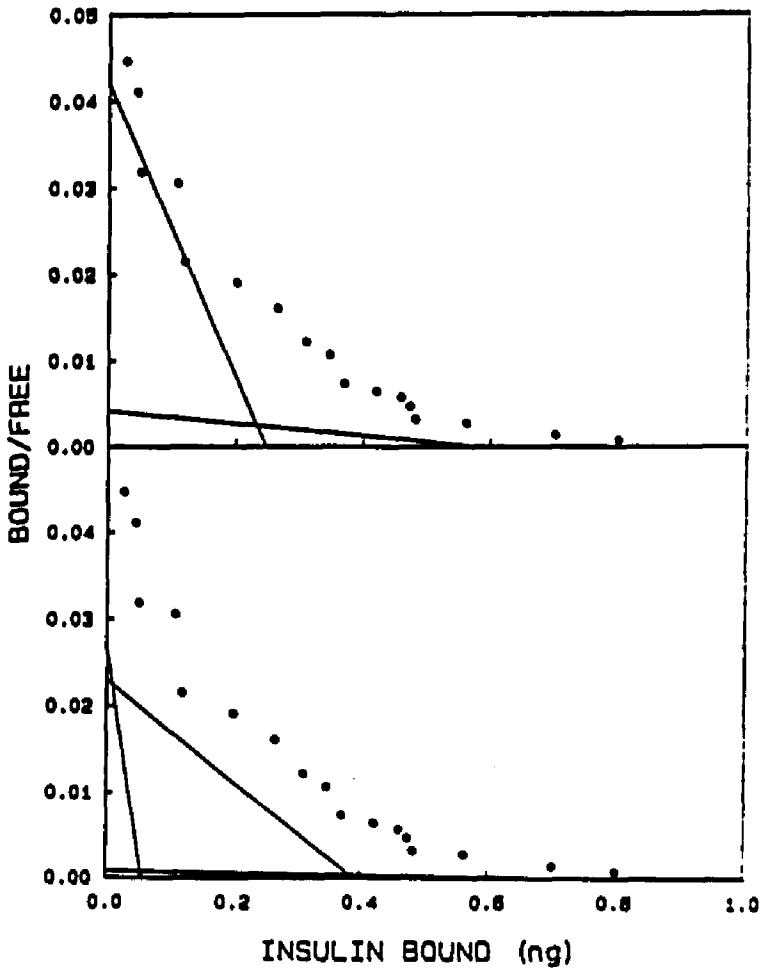

Figure 17. Curve fitting of a Scatchard plot of insulin binding to rat liver microsomes. Comparison of the two-site model (upper panel) with the three-site model (lower panel) for the same data. These plots are representative of those used to calculate the average K_a and R values for liver shown in Table 8.

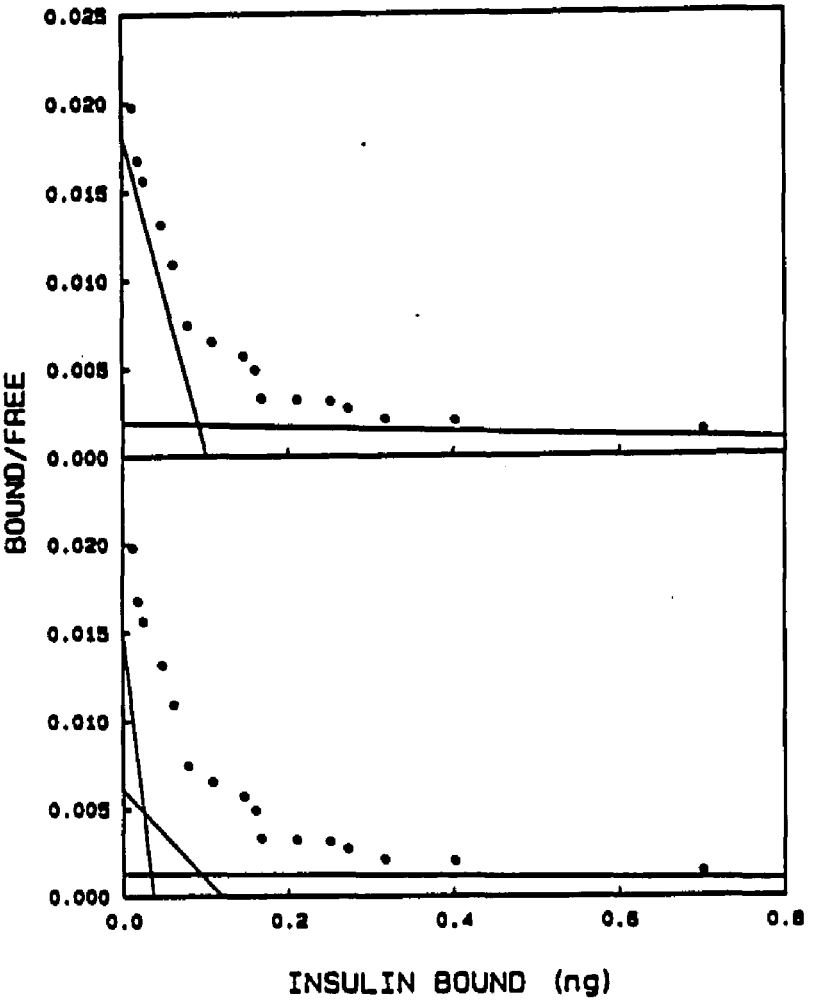

Figure 18. Curve fitting of the Scatchard plot of insulin binding to rat mammary microsomes. Comparison of the two-site model (upper panel) with the three-site model (lower panel) for the same data. These plots are representative of those used to calculate the average K_a and R values for mammary gland shown in Table 8.

model was employed (upper panels). The K_a and R estimates of the medium and low affinity sites determined using the three site model were similar to those of the high and low affinity sites determined using the two site model (Table 8). Thus, the high affinity binding site of the three site model was obscured by the medium affinity binding site when the two site model was used to fit the binding data.

The insulin affinity constants reported here for the medium and low affinity sites of the three site model and the high and low affinity sites of the two site model correspond to affinity constants previously determined for insulin binding to rat liver and fat cell membranes using a two site model.[15] Assuming three classes of insulin receptors, Marinetti et al[25] reported an affinity constant of $1.0 \times 10^{10} M^{-1}$ for the high affinity receptor of rat liver membranes while Oscar et al[26] reported a constant of $5.9 \times 10^{10} M^{-1}$ for bovine mammary membranes. Our mean constants for the high affinity receptor of rat liver and mammary microsomes ($5.6 \times 10^{14} M^{-1}$ and $2.8 \times 10^{13} M^{-1}$, respectively) were considerably higher than those reported by Marinetti et al[25] and Oscar et al.[26] However, the affinity constants for this site were unusually large

Table 8. Overall Means for Rat Liver and Mammary Receptor Variables - Two vs Three Site Model.

	Liver (13)*		Mammary Gland (12)*	
$K_a(M^{-1})$**	2 Site Model##	3 Site Model##	2 Site Model##	3 Site Model##
High Affinity Site	—	5.6×10^{14} (5.1×10^{14})	—	2.8×10^{13} (1.9×10^{13})
Medium Affinity Site	7.2×10^{8} (4.6×10^{7})	4.9×10^{8} (4.1×10^{7})	7.8×10^{8} (8.3×10^{7})	2.0×10^{8} (2.6×10^{7})
Low Affinity Site	9.2×10^{6} (3.0×10^{6})	3.6×10^{6} (8.0×10^{5})	1.4×10^{7} (3.5×10^{6})	3.6×10^{6} (7.5×10^{5})
R(moles per 500 µg Lowry protein)#				
High Affinity Site	—	4.5×10^{-15} (1.3×10^{-15})	—	3.4×10^{-15} (1.1×10^{-15})
Medium Affinity Site	9.3×10^{-14} (7.6×10^{-15})	1.1×10^{-13} (7.4×10^{-15})	2.1×10^{-14} (3.0×10^{-15})	5.0×10^{-14} (6.6×10^{-15})
Low Affinity Site	5.5×10^{-13} (9.5×10^{-14})	1.1×10^{-12} (3.1×10^{-13})	3.7×10^{-13} (5.0×10^{-14})	7.7×10^{-13} (1.0×10^{-13})

* Numbers in parentheses = number of animals.
** K_a = affinity constant.
\# R = insulin binding capacity.
\#\# Values in parentheses are SEM.

($10^{13}<K_a<10^{16}$) for four of our liver and two of our mammary preparations. Examination of the Scatchard plots for these microsomal preparations showed that the high affinity portion of the curve was poorly defined because of too few data points to accurately estimate K_a and R. Elimination of these preparations from the analysis resulted in mean affinity constants of $2.0 \pm 1.0 \times 10^{10} M^{-1}$ and $1.1 \pm 0.4 \times 10^{10} M^{-1}$ for liver and mammary microsomes, respectively. Hence, the affinity constants of the high affinity receptor for the majority of liver and mammary preparations examined in this study were similar to those reported by Marinetti et al[25] and Oscar et al.[26]

The effect of diet on the binding parameters (K_a and R) of liver and mammary microsomes estimated using the three site model is presented in Table 9. Note, the three mammary samples exhibiting only two receptor sites were excluded from the mammary analyses. Also, those tissue preparations exhibiting unusually high K_a values for the high affinity site were not eliminated from the analyses since elimination of these preparations would have made statistical analysis impossible due to inadequate sample size. Diet had no effect on the insulin binding capacities ($P>0.10$) or binding affinities ($P>0.10$) of the three receptor sites in mammary microsomes. With respect to

Table 9. Effect of Diet on the Binding Capacities and Binding Affinities of Insulin in Rat Liver and Mammary Microsomes - Three Site Model.#,##

Measurement**	Diet*				
Liver	Control	HF-LC	HC-LF	SEM+	Significance++
n	3	5	5		
K_{a1}	13.218 ($1.7x10^{13}$)	11.588 ($3.9x10^{11}$)	9.643 ($4.4x10^{9}$)	0.723	NS
K_{a2}	8.796[a] ($6.2x10^{8}$)	8.713[a] ($5.2x10^{8}$)	8.562[b] ($3.6x10^{8}$)	0.029	P<0.05
K_{a3}	6.748 ($5.6x10^{6}$)	6.263 ($1.8x10^{6}$)	6.141 ($1.4x10^{6}$)	0.154	NS
R_1	$3.0x10^{-15}$[ab]	$1.3x10^{-15}$[a]	$9.8x10^{-15}$[b]	$1.3x10^{-15}$	P<0.10
R_2	$1.1x10^{-13}$	$1.0x10^{-13}$	$1.1x10^{-13}$	$9.8x10^{-15}$	NS
R_3	$4.0x10^{-13}$	$1.2x10^{-12}$	$1.4x10^{-12}$	$4.5x10^{-13}$	NS
Mammary					
n	1	3	5		
K_{a1}	10.165 ($1.5x10^{10}$)	10.757 ($5.7x10^{10}$)	10.304 ($2.0x10^{10}$)	0.943	NS
K_{a2}	8.377 ($2.4x10^{8}$)	8.084 ($1.2x10^{8}$)	8.268 ($1.9x10^{8}$)	0.091	NS
K_{a3}	7.074 ($1.2x10^{7}$)	6.038 ($1.1x10^{6}$)	6.329 ($2.1x10^{6}$)	0.124	NS
R_1	$8.2x10^{-15}$	$6.7x10^{-16}$	$3.7x10^{-15}$	$2.0x10^{-15}$	NS
R_2	$3.2x10^{-14}$	$5.5x10^{-14}$	$5.8x10^{-14}$	$8.2x10^{-15}$	NS
R_3	$1.7x10^{-13}$	$1.1x10^{-12}$	$9.0x10^{-13}$	$1.7x10^{-13}$	NS

* HF-LC = high fat-low carbohydrate diet; HC-LF = high carbohydrate-low fat diet

** n = number of animals; K_{a1} = binding affinity of the high affinity site; K_{a2} = binding affinity of the medium affinity site; K_{a3} = binding affinity of the low affinity site; R_1 = binding capacity of the high affinity site, R_2 = binding capacity of the medium affinity site, R_3 = binding capacity of the low affinity site.

#Binding affinities are listed as log K_a and as $K_a(M^{-1})$ directly below in parentheses; Binding capacities are moles insulin bound/500 µg Lowry protein.

##Values are least squares means.

+SEM = standard error of the mean. SEM listed for K_a are in terms of log K_a.

++NS = not significant. Means in a row not sharing a common superscript are significantly different.

liver microsomes, the HC-LF group exhibited a lower binding affinity for the medium affinity binding site (K_{a2}) than did the HF-LC and control groups. Also, the HC-LF group had a higher binding capacity for the high affinity binding site (R_1) than did the HF-LC group; however, R_1 of the HF-LC group was not different from R_1 of the control group. All other liver parameters were not influenced by diet ($P>0.10$).

Of interest is the fact that liver microsomes of the HF-LC and control groups had similar R_1 values. Previous studies[1,2] reported that high fat diets decreased insulin binding to rat adipocytes and liver plasma membranes by decreasing the number of insulin receptors, but those studies did not include a control diet in their analysis. Instead, a high carbohydrate diet was used for comparison. Our results raise the question, does the high fat diet actually decrease insulin binding or does the high carbohydrate diet increase it?

Failure to detect any dietary effects regarding insulin binding to rat mammary microsomes may have been due to the limited sample sizes of both the control and HF-LC groups. Conversely, the dietary differences detected in K_{a2} and R_1 of rat liver microsomes may have resulted from

including all liver preparations in the analysis. As mentioned previously, the high affinity portion of four of the Scatchard plots for liver were poorly defined; therefore, inclusion of these samples in the analysis could have erroneously resulted in significance.

Numerous factors other than multiple classes of binding sites can result in nonlinear Scatchard plots.[27] These factors are: 1) a high degree of nonspecific binding; 2) different binding affinities of labeled and unlabeled ligand for the receptor; 3) differential rates of degradation of native vs labeled ligand; 4) cooperativity among binding sites; 5) heterogeneity of labeled ligand; 6) ligand-ligand interactions; 7) inactivation or chemical transformation of ligand or receptor during the binding reaction; and 8) binding reactions other than the simple bimolecular reaction, A + B = AB.[27]

Insulin contains four potential iodination sites, tyrosine residues A14, A19, B16, and B26.[28] Biological activity and binding affinity of iodinated insulin vary depending on the position of the label, monoiodo-(A19)-insulin being relatively less potent and monoiodo-(B16 and B26)-insulins being relatively more potent than monoiodo-(A14)-insulin.[28] Use of heterogeneously-labeled preparations of insulin (factor 5 above) containing significant

quantities of monoiodo-(B26)-insulin can result in the appearance of a high affinity receptor component in the Scatchard plot.[29] Since the distribution of ^{125}I-labeling in our ^{125}I-insulin preparation was not determined, it is possible that the high affinity binding site observed in our studies is due to ligand heterogeneity. However, ligand heterogeneity does not explain why we failed to detect this high affinity site in three of our mammary preparations because assay conditions for all tissue preparations, including the ^{125}I-insulin preparation employed, were held constant.

Oscar et al[26] reported three classes of insulin receptors in bovine mammary microsomes; however, they detected only two classes of insulin receptors using the conventional competitive binding assay and two site Scatchard analysis. Both classes of receptors were similar in binding affinities (K_{a1} = 3.1 x $10^{9}M^{-1}$ and K_{a2} = 6.3 x $10^{7}M^{-1}$) to those reported previously for a number of tissues and species.[15] Using a direct titration assay,[30] these investigators[26] again detected two classes of insulin receptors, but the binding affinities of these sites differed from those detected via the competitive binding assay. The binding affinity of the high affinity site detected by the direct titration method was 17-fold higher

than that of the high affinity site detected using the competitive binding assay. The binding affinity of the low affinity site detected by direct titration was similar to that of the high affinity site detected by the competitive binding assay. They concluded that their competitive binding assay was not sensitive enough to detect the high affinity site determined by direct titration. Their results support our proposal that the high affinity insulin binding site detected with the two site model is equivalent to the medium affinity site detected with the three site model.

The inability of Oscar et al[26] to detect the highest affinity site using the competitive binding assay may have resulted from a lack of data points in this critical region of the curve. Kahn[15] indicated that Scatchard plots can be misinterpreted if the binding data are incomplete. Several investigators[16-18] have erroneously reported a single class of insulin receptors because they failed to analyze the entire binding curve. Kono and Barham,[16] analyzing only the low affinity portion of the Scatchard curve, reported a binding affinity of $2 \times 10^{8} M^{-1}$. Conversely, Cuatrecasas,[17,18] analyzing only the high affinity portion of the Scatchard curve, reported a binding affinity of $2 \times 10^{10} M^{-1}$. Our ability to detect

three receptor sites was facilitated by the inclusiveness and range of our binding data. Despite the inclusiveness of our data, the high and low affinity regions of our curves were still somewhat deficient in data points resulting in an inability to accurately describe K_a and R for these sites. Nevertheless, the qualitative existence of three insulin binding sites was verified by both visual and mathematical means.

Biological significance is usually attributed only to the high affinity-low capacity site in the heterogeneous receptor model of insulin binding.[25,31] However, Kono[32] has disputed this concept on the basis that certain biological effects of insulin occur only at higher insulin concentrations. Furthermore, Freychet et al[33] demonstrated that insulin analogues displace ^{125}I-insulin from the low affinity-high capacity receptor site in order of their bioactivity. Therefore, it would appear that insulin may specifically bind to these low affinity sites to exert certain biological effects.

It is well established that the insulin-like growth factors (IGFs) share structural and biological similarities with insulin.[34] IGFs mimic the effects of insulin in adipose tissue by stimulating glucose transport as well as glycogen, protein and lipid synthesis and by inhibiting

lipolysis.[35] In muscle, the insulin-like effects of IGFs include enhancement of glucose transport, glycolysis, and glycogen and protein synthesis.[35]

To date, two types of IGF receptors have been structurally identified, the Type I receptor, which binds IGF I with greater affinity than IGF II, and the Type II receptor, which binds IGF II with greater affinity than IGF I.[36] The Type I receptor is structurally similar to the insulin receptor in molecular weight and subunit composition[36] and binds insulin at high insulin concentrations (K_a = 6.0 - 8.9 x $10^6 M^{-1}$).[37,38] The Type II receptor appears structurally unrelated to the insulin receptor which may account for its inability to bind insulin.[36] Additional types of IGF receptors have been postulated because the patterns of binding crossreactivity cannot be explained solely on the basis of two receptor types.[34] Recently, Hintz et al[39] characterized a third type of IGF receptor in human lymphoid cells and placental membranes. This Type III IGF receptor had approximately equal affinity for IGF I and II (K_a = 1.7 x $10^9 M^{-1}$ and 1.2 x $10^9 M^{-1}$, respectively) and only slightly less affinity for insulin (K_a = 3 x $10^8 M^{-1}$). Interestingly, the affinity constants reported in our study for the medium and low affinity insulin receptors of liver (K_a = 4.9 x $10^8 M^{-1}$ and

$3.6 \times 10^6 M^{-1}$, respectively) and mammary tissue ($K_a = 2.0 \times 10^8 M^{-1}$ and $3.6 \times 10^6 M^{-1}$, respectively) correspond closely with the affinity constants reported for insulin binding to the Type III[32] and Type I[37,38] IGF receptors ($K_a = 3 \times 10^8 M^{-1}$ and $6.0 - 8.9 \times 10^6 M^{-1}$, respectively).

Campbell and Baumrucker[40] recently characterized IGF I receptors in bovine mammary microsomes using ^{125}I-recombinant human somatomedin C. Furthermore, Type I receptors have been identified in certain cell lines of rat liver (BRL-3A2).[41,42] The presence of Type I IGF receptors in rat liver and mammary preparations in addition to the recent characterization of a Type III receptor in human lymphoid and placental preparations raises the interesting possibility that the medium and low affinity insulin binding sites described in this report may represent Type III and Type I IGF receptors, respectively. Clearly, a broad investigation of insulin and IGF binding to mammary and liver cell receptors, as well as isolation and identification of the proposed receptor types, will be required to establish biological significance of these receptor sites.

In conclusion, diet had no effect on the insulin binding parameters of rat liver and mammary microsomes derived using the two site model. However, a two site

model did not accurately describe the insulin binding data. Instead, a three site model was superior in fitting the insulin binding data of all rat liver and nine of twelve rat mammary microsomal preparations. Although the appearance of a high affinity site in these studies may have resulted from the use of heterogeneously-labeled ^{125}I-insulin, this seems unlikely since the two site model was superior in fitting the data of three of the mammary preparations. Uncertainty in fitting the high affinity site using the three site model for six of the tissue preparations (four liver and two mammary) was due to a lack of data points in this region of the curve. Analysis of the liver and mammary binding parameters estimated with the three site model resulted in significant dietary effects for only R_1 and K_{a2} of rat liver. Therefore, a more thorough examination of insulin binding in the concentration range of $0 - 5 \times 10^{-10}$M (0 - 3 ng/ml) is needed to accurately describe the quantitative aspects of insulin binding to this site. Furthermore, dietary studies employing more animals per treatment are needed before definitive conclusions can be made concerning the effect of dietary fat on insulin receptors in rat liver and mammary gland.

LIST OF REFERENCES

1. Ip C, Tepperman HM, Holohan P, et al: Insulin binding and insulin response of adipocytes from rats adapted to fat feeding. J Lipid Res 17:588-599, 1976

2. Sun JV, Tepperman HM, Tepperman J: A comparison of insulin binding by liver plasma membranes of rats fed a high glucose diet or a high fat diet. J Lipid Res 18:533-539, 1977

3. Wahle KWJ: Fatty acid modification and membrane lipids. Proc Nutr Soc 42:273-287, 1983

4. Berlin E, Bhathena SJ, Kliman PG, et al: Effect of saturation of dietary lipids on insulin receptors and membrane fluidity in rabbits. Fed Proc 44:1236, 1985 (Abstr)

5. Banks W, Clapperton JL, Ferrie ME, et al: Effect of feeding fat to dairy cows receiving a fat-deficient basal diet. J Dairy Res 43:213-218, 1976

6. MacLeod GK, Yu Y, Schaeffer LR: Feeding value of protected animal tallow for high yielding dairy cows. J Dairy Sci 60:726-738, 1977

7. Storry JE, Brumby PE, Hall AJ, et al: Response of the lactating cow to different methods of incorporating casein and coconut oil in the diet. J Dairy Sci 57:61-67, 1974

8. Palmquist DL, Moser EA: Dietary fat effects on blood insulin, glucose utilization, and milk protein contents of lactating cows. J Dairy Sci 64:1664-1670, 1981

9. Guyton AC: Textbook of Medical Physiology (ed 5). Philadelphia, WB Saunders Co, 1976, p 1042

10. Scatchard G: The attractions of proteins for small molecules and ions. Ann N Y Acad Sci 51:660-672, 1949

11. Olefsky JM, Ciaraldi TP: The insulin receptor: Basic characteristics and its role in insulin resistant

states, in Brownlee M (ed): Diabetes Mellitus. New York, Garland STPM, 1981, pp 73-116

12. Kahn CR: Membrane receptors for hormones and neurotransmitters. J Cell Biol 70:261-286, 1976

13. Pollet RJ, Levey, GS: Principles of membrane receptor physiology and their application to clinical medicine. J Cardiovascular Med 92:663-680, 1980

14. Smith DH, Palmquist DL, Schanbacher FL: Characterization of insulin binding to bovine liver and mammary microsomes. Comp Biochem Physiol (in press), 1986

15. Kahn CR: Membrane receptors for polypeptide hormones, in Korn ED (ed): Methods in Membrane Biology, vol 3. New York, Plenum, 1975, pp 81-146

16. Kono T, Barham FW: The relationship between the insulin-binding capacity of fat cells and the cellular response to insulin. Studies with intact and trypsin-treated fat cells. J Biol Chem 246:6210-6216, 1971

17. Cuatrecasas P: Insulin receptor interactions in adipose tissue cells: Direct measurement and properties. Proc Natl Acad Sci 68:1264-1268, 1971

18. Cuatrecasas P: Properties of the insulin receptor of isolated fat cell membranes. J Biol Chem 246:7265-7274, 1971

19. Lavau M, Fried SK, Susini C, et al: Mechanism of insulin resistance in adipocytes of rats fed a high-fat diet. J Lipid Res 20:8-16, 1979

20. Lowry, OH, Rosebrough NJ, Farr AL, et al: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275, 1951

21. Freychet P, Roth J, Neville DM Jr: Monoiodoinsulin: Demonstration of its biological activity and binding to fat cells and liver membranes. Biochem Biophys Res Commun 43:400-408, 1971

22. Metzler CM, Elfring GL, McEwen AJ: A package of computer programs for pharmacokinetic modeling. Biometrics 30:562-563, 1974

23. Harvey WR: Least squares analysis of data with unequal subclass numbers. USDA Res Ser ARS-20-80, 1960

24. Steel RGD, Torrie JH: Principles and Procedures of Statistics (ed 2). New York, McGraw-Hill, 1980, pp 187-188

25. Marinetti GV, Schlatz L, Reilly K: Hormone-membrane interactions, in Fritz I B (ed): Insulin Action. New York, Academic, 1972, pp 207-276

26. Oscar TP, Baumrucker CR, Etherton TD: Insulin binding to bovine mammary membranes: Comparison of microsomes versus smooth membranes. J Anim Sci 62:179-186, 1986

27. Cuatrecasas P, Hollenberg MD: Membrane receptors and hormone action, in Anfinsen CB, Edsall JT, Richards FM (eds): Advances in Protein Chemistry, vol 30. New York, Academic, 1976, pp 251-451

28. Gammeltoft S: Insulin receptors: Binding kinetics and structure - function relationship of insulin. Physiol Rev 64:1321-1378, 1984

29. Taylor SI: Binding of hormones to receptors. An alternative explanation of nonlinear Scatchard plots. Biochemistry 14:2357-2361, 1975

30. Jacobs S, Chang KJ, Cuatrecasas P: Estimation of hormone receptor affinity by competitive displacement of labeled ligand: Effect of concentration of receptor and labeled ligand. Biochem Biophys Res Commun 66:687-692, 1975

31. Kahn CR, Freychet P, Roth J, et al: Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. J Biol Chem 249:2249-2257, 1974

32. Kono T: The insulin receptor of fat cells: The relationship between the binding and physiological effects of insulin, in Fritz IB (ed): Insulin Action. New York, Academic,1972, pp 171-203

33. Freychet P, Roth J, Neville DM Jr: Insulin receptors in the liver: Specific binding of [^{125}I] insulin to the plasma membrane and its relation to insulin bioactivity. Proc Natl Acad Sci USA 68:1833-1837, 1971

34. Hall K, Sara VR: Growth and somatomedins. Vit Horm 40:175-233, 1983

35. Froesch ER, Schmid C, Schwander J, et al: Actions of insulin-like growth factors. Ann Rev Physiol 47:443-467, 1985

36. Rechler MM, Nissley SP: The nature and regulation of the receptors for insulin-like growth factors. Ann Rev Physiol 47:425-442, 1985

37. Bar RS, Boes M: Distinct receptors for IGF-I, IGF-II, and insulin are present on bovine capillary endothelial cells and large vessel endothelial cells. Biochem Biophys Res Commun 124:203-209, 1984

38. Polychronakos C, Guyda HJ, Posner BI: Receptors for the insulin-like growth factors on human erythrocytes. J Clin Endocrinol Metab 57:436-438, 1983

39. Hintz RL, Thorsson AV, Enberg G, et al: IGF-II binding on human lymphoid cells: Demonstration of a common high affinity receptor for insulin like peptides. Biochem Biophys Res Commun 118:774-782, 1984

40. Campbell PG, Baumrucker CR: Characterization of insulin-like growth factor-1/somatomedin C receptors in the bovine mammary gland. J Dairy Sci 69:163, 1986 (abstr)

41. Rechler MM, Zapf J, Nissley SP, et al: Interactions of the insulin-like growth factors I and II and multiplication-stimulating activity with receptors and serum carrier proteins. Endocrinology 107:1451-1459, 1980

42. Zick Y, Sasaki N, Rees-Jones RW, et al: Insulin-like growth factor-I (IGF-I) stimulates tyrosine kinase activity in purified receptors from a rat liver cell line. Biochem Biophys Res Commun 119:6-13, 1984

CHAPTER VI

CONCLUSIONS

Insulin receptors were identified in bovine mammary microsomes. These receptors were similar to insulin receptors in other tissues and species with respect to pH optimum, binding affinity, and binding specificity. Scatchard plots of the insulin binding data for bovine mammary microsomes were curvilinear, indicating the presence of two classes of insulin receptors, a high affinity-low capacity site and a low affinity-high capacity site.

If the high affinity-low capacity site represents the biological insulin receptor as has been postulated (9, 15), insulin receptor occupancy would exceed 50% at most physiological insulin concentrations. Extremely low plasma insulin concentrations, as seen during starvation, would still result in 26% loading of bovine mammary insulin receptors. Since the "spare receptor" theory of insulin action (20) maintains that receptor occupancy of only 10% is required for maximal stimulation of the cell, bovine mammary epithelial cells ought to be under maximal insulin stimulation at all times. Hence, insulin receptor

regulation in bovine mammary tissue would be of questionable physiological significance.

Conversely, not all insulin-sensitive metabolic pathways necessarily conform to the "spare receptor" theory. Some insulin-sensitive processes, such as amino acid uptake in rat hepatocytes (3), respond in a dose-dependent fashion. Moreover, a baseline level of 10% insulin receptor occupancy for maximal cellular stimulation is somewhat arbitrary since the level of receptor loading required to elicit the maximal cellular response varies as a function of the metabolic pathway (3, 19), species (19, 25), or tissue (13) in question. Therefore, a regulatory role for the insulin receptor in mammary metabolism cannot be ruled out on the basis of receptor occupancy levels alone. Coordinated studies examining the effect of insulin on glucose, lipid, and protein metabolism in bovine mammary cells or alveoli as well as insulin binding to these preparations will be necessary to determine the relationship between insulin binding and subsequent physiological action in the mammary gland.

Kono (34) has disputed that the high affinity insulin binding site is the biological insulin receptor on the basis that certain biological effects of insulin occur only at high insulin concentrations. This raises the

interesting possibility that both classes of insulin receptors are involved in insulin action, but that each mediates different effects of insulin. With respect to the mammary cell, the high affinity site could be responsible for the maintenance role of insulin in the gland. Since the high affinity site would be saturated at all insulin and insulin receptor concentrations, this site would be fully activated at all times, i.e., no gradation in response would be apparent. This could be a metabolic adaptation of the gland to ensure maximal activity of metabolic pathways vital to the maintenance of milk secretion, such as glucose transport.

On the other hand, the low affinity site could be responsible for the regulatory role of insulin in mammary metabolism. Jones et al. (8) reported that a small physiological increase in plasma insulin concentration resulted in a significant increase in mammary lipogenesis in the rat. In view of the low K_a value of the low affinity receptor, it seems unlikely that the slight increase in receptor occupancy resulting from such a small rise in plasma insulin could account for the degree of increased responsiveness observed in this study. Hence, it does not appear likely that the low affinity site is responsible for the regulatory role of insulin in the gland.

Assuming that the high affinity insulin binding site is the biological insulin receptor, both maintenance and regulatory effects of insulin must be mediated via the same binding site. This suggests that a higher level of control exists beyond the receptor itself. To date, two putative second messengers for the insulin receptor have been identified, a tyrosine kinase which is intrinsic to the insulin receptor (23) and a low molecular weight polypeptide which is released from the plasma membrane (24). Both mediators appear to function differently, the kinase via phosphorylation reactions (23) and the polypeptide via dephosphorylation reactions (24). It is possible that one of these messengers is responsible for the maintenance effects of insulin in the cell while the other is responsible for the regulatory effects. If this is the case, some mechanism must be operating whereby insulin binding to the receptor results in differential generation of each messenger. Differential messenger generation could be linked to receptor occupancy level and/or structural arrangement of receptors in the plasma membrane. Examination of the relationship between insulin receptor occupancy and second messenger generation, as well as identification of those metabolic pathways influenced by

each messenger will be required to verify a dual mode of insulin action in mammary cells.

Scatchard plots of the insulin binding data for rat mammary microsomes, like those for bovine mammary microsomes, were curvilinear suggesting the presence of multiple classes of insulin binding sites. However, use of the NONLIN program (17) to fit the binding data of rat mammary microsomes indicated the presence of three rather than two classes of insulin receptors. Since a three site model was not employed to fit the binding data of bovine mammary microsomes, it is possible that the three site model may have provided a better fit than the two site model. Oscar et al. (21) reported three classes of insulin receptors in bovine mammary microsomes; hence, a third class of insulin receptors may have been present in our preparations which went undetected when the two site model was employed.

The binding affinities of the medium and low affinity binding sites in rat mammary microsomes corresponded closely with the binding affinity of insulin for the Type III and Type I insulin-like growth factor (IGF) receptors, respectively. Campbell and Baumrucker (2) have identified Type I receptors in bovine mammary microsomes. Therefore, the medium and low affinity binding sites for insulin in

rat mammary microsomes, as well as the low affinity binding site for insulin in bovine mammary microsomes may represent IGF receptors.

IGFs, as the name implies, share many biological similarities with insulin. Like insulin, IGFs have been shown to stimulate glucose transport, glycolysis, glycogenesis, lipogenesis, and protein synthesis (5). If IGF receptors are present in the mammary gland, it is possible that some of the effects normally attributed to insulin in this tissue are, in fact, caused by IGFs. For example, insulin has been shown to stimulate amino acid uptake (14), as well as synthesis of casein (18), casein mRNA (1), and α-lactalbumin (18) by the rodent mammary gland. However, the concentration of insulin required for these stimulatory effects is 20-5000 fold more than the physiological concentration of insulin in ruminants and nonruminants. Since insulin binds to the Type I IGF receptor at high insulin concentrations, the stimulatory effect of insulin on mammary protein metabolism may be mediated via the Type I receptor. With this in mind, the possibility exists that the regulatory role of insulin in mammary metabolism is mediated via an IGF receptor, and

hence, is attributable to IGFs rather than insulin. Identification of IGF receptors in the mammary gland plus differentiation between the metabolic effects of insulin and IGF in this tissue are necessary if insulin's role in mammary metabolism is to be fully understood.

Specific insulin binding to bovine milk fat globule membranes (MFGM) was similar to that seen in freeze-thawed bovine mammary microsomes. Since specific insulin binding to bovine mammary microsomes is indicative of an insulin receptor in bovine mammary tissue, it seems reasonable to conclude that specific insulin binding to bovine MFGM is also indicative of an insulin receptor. This proposal is further supported by the presence of insulin receptors in caprine and murine MFGM (4).

Insulin receptors in MFGM probably reflect insulin receptors in the apical plasma membrane of the mammary epithelial cell as MFGM are derived from the apical plasma membrane (16). However, the origin and/or function of insulin receptors in the apical plasma membrane is unknown. In the mammary cell, insulin is internalized rapidly via a receptor-mediated process (8), although the fate of the internalized insulin-receptor complex has yet

to be determined. In most cells, the internalized complexes localize at the lysosomes where insulin, the receptor, or both are degraded (22). But in the actively-secreting mammary epithelial cell, lysosomal activity seems to be minimal (6, 7). Recent evidence (10) suggests that the insulin receptor may serve as a transport protein in bovine endothelial cells, translocating intact insulin unidirectionally across the cell from the blood to the extracellular space. The mammary cell, like the endothelial cell, may translocate insulin-receptor complexes unidirectionally from the basolateral to the apical plasma membrane. Such receptor trafficking could account for insulin (12) and insulin receptors in milk and might represent a simple alternative to lysosomal degradation of the complex. Furthermore, if unidirectional translocation of insulin receptors occurs as decribed, the insulin receptor of the apical plasma membrane would reflect insulin receptors of the basolateral membranes; therefore, MFGM could be used to monitor the insulin receptor status of the mammary gland.

If the scheme of insulin receptor trafficking proposed above is valid, the fact that insulin receptors in

MFGM are regulated by physiological and nutritional factors suggests that the gland may be able to regulate insulin action by regulating the flow of insulin-receptor complexes. Regulation of insulin-receptor complex turnover may represent one means by which the mammary cell could modulate its insulin responsiveness independent of insulin receptor numbers and/or binding affinity.

Although some of the insulin receptors in the apical plasma membrane, and hence MFGM, seem to represent translocated basolateral receptors, not all receptors in the apical plasma membrane are necessarily of basolateral origin. Golgi-derived secretory vesicles are thought to replenish the apical plasma membrane depleted during milk fat secretion (16). In addition, insulin receptors have been identified in the Golgi cisternae, implicating this organelle in insulin receptor synthesis (22). Therefore, insulin binding sites in the apical plasma membrane may represent newly synthesized insulin receptors. A biological function for the insulin receptor at the apical surface of the mammary epithelial cell seems improbable since insulin is present in milk (12-115 μU/ml) (12) and could potentially trigger intracellular events through an apical membrane receptor. Therefore, the secreting gland would be self stimulating for insulin, a puzzling state if real.

The presence of insulin receptors in bovine MFGM, and hence, the apical plasma membrane of the mammary epithelial cell, raises numerous questions: what is the origin of the insulin receptor in the apical plasma membrane; do they accurately reflect the insulin receptor status of the mammary cell proper; is insulin degraded appreciably by the mammary epithelial cells; are insulin-receptor complexes transported unidirectionally from the basolateral to the apical plasma membrane; is the MFGM insulin receptor similar structurally to the receptor of the basolateral membrane; and can milk insulin trigger intracellular events via the insulin receptor of the apical plasma membrane? Answers to these questions will be needed to establish the biological role of the insulin receptor in MFGM, to verify the usefulness of MFGM in monitoring insulin receptor regulation by the mammary cell, and to further our understanding of polypeptide hormone receptor trafficking in the mammary cell.

Finally, it must be stressed that insulin receptor numbers in microsomal preparations do not necessarily reflect insulin receptor numbers in the plasma membrane. Regulation of plasma membrane insulin receptors may go undetected if a microsomal preparation is employed since

modulation of receptors may occur via cellular redistribution of receptors rather than via changes in receptor synthesis or degradation.

While the studies reported here have provided a foundation of knowledge concerning the insulin receptor in mammary metabolism, the results of this work have raised more questions than they have answered. Moreover, the question of insulin receptor regulation in the mammary gland was never resolved since a cellular system is imperative to accurately monitor receptor modulation in the plasma membrane.

At the time this dissertation problem was initiated, cell isolation procedures and tissue culture methodology for bovine mammary cells and alveoli had not been perfected. For this reason, bovine mammary microsomes were employed in these studies. However, functional bovine mammary cells and alveoli are now routinely prepared by several laboratories, including our own. Research employing these mammary preparations will be necessary to clarify the role of insulin in mammary metabolism from the standpoint of insulin receptor regulation. These cellular and alveolar preparations will also serve as a useful research tool for advancing our understanding of the hormonal and metabolic control of lactation in the cow.

LIST OF REFERENCES

1. Bolander, F. F., Jr., K. R. Nicholas, J. J. Van Wyk, and Y. J. Topper. 1981. Insulin is essential for accumulation of casein mRNA in mouse mammary epithelial cells. Proc. Natl. Acad. Sci. USA 78:5682.

2. Campbell, P. G. and C. R. Baumrucker. 1986. Characterization of insulin-like growth factor-1/somatomedin C receptors in the bovine mammary gland. J. Dairy Sci. 69(Suppl. 1):163. (Abstr.)

3. Dolais-Kitabgi, J., F. Checler, J. Rey, O. Morin, and P. Freychet. 1981. Insulin stimulation of amino acid transport in primary cultured rat hepatocytes varies in direct proportion to insulin binding. FEBS Letters 128:321.

4. Flint, D. J. and D. W. West. 1983. Insulin receptors in milk. Br. Vet. J. 139:39.

5. Hall, K. and V. R. Sara. 1983. Growth and somatomedins. Vit. Horm. 40:175.

6. Helminen, H. J. and J. L. E. Ericsson. 1970. Quantitation of lysosomal enzyme changes during enforced mammary gland involution. Exper. Cell Res. 60:419.

7. Helminen, H. J. and J. L. E. Ericsson. 1971. Effects of enforced milk stasis on mammary gland epithelium, with special reference to changes in lysosomes and lysosomal enzymes. Exper. Cell Res. 68:411.

8. Jones, R. G., V. Ilic, and D. H. Williamson. 1984. Physiological significance of altered insulin metabolism in the conscious rat during lactation. Biochem. J. 220:455.

9. Kahn, C. R., P. Freychet, J. Roth, and D. M. Neville, Jr. 1974. Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. J. Biol. Chem. 249:2249.

10. King, G. L. and S. M. Johnson. 1985. Receptor-mediated transport of insulin across endothelial cells. Science 227:1583.

11. Kono, T. 1972. The insulin receptor of fat cells: The relationship between the binding and physiological effects of insulin. Pages 171-203 in Insulin action. I. B. Fritz, ed. Academic Press, New York, NY.

12. Kulski, J. K. and P. E. Hartman. 1983. Milk insulin, GH and TSH: Relationship to changes in milk lactose, glucose and protein during lactogenesis in women. Endocrin. Exper. 17:317.

13. LeMarchand, Y., E. G. Loten, F. Assismacoponlos-Jeannet, M. Forgue, P. Freychet, and B. Jeanrenaud. 1977. Effects of fasting and streptozotocin in the obese-hyperglycemic (ob/ob) mouse. Diabetes 26:582.

14. Lobitz, C. J. and M. C. Neville. 1977. Control of amino acid transport in the mammary gland of the pregnant mouse. J. Supramol. Struct. 6:355.

15. Marinetti, G. V., L. Schlatz, and K. Reilly. 1972. Hormone-membrane interactions. Pages 207-276 in Insulin action. I. B. Fritz, ed. Academic Press, New York, NY.

16. Mather, I. H. and T. W. Keenan. 1983. Function of endomembranes and the cell surface in the secretion of organic milk constituents. Pages 232-283 in Biochemistry of lactation. T. B. Mepham, ed. Elsevier Science Publishers, BV, New York, NY.

17. Metzler, C. M., G. L. Elfring, and A. J. McEwen. 1974. A package of computer programs for pharmacokinetic modeling. Biometrics 30:562.

18. Nicholas, K. R. and Y. J. Topper. 1983. Anti-insulin receptor serum mimics the developmental role of insulin in mouse mammary explants. Biochem. Biophys. Res. Commun. 111:988.

19. Olefsky, J. M. 1975. Effect of dexamethasone on insulin binding, glucose transport, and glucose oxidation of isolated rat adipocytes. J. Clin. Invest. 56:1499.

20. Olefsky, J. M. and T. P. Ciaraldi. 1981. The insulin receptor: Basic characteristics and its role in insulin resistant states. Pages 73-116 in Diabetes mellitus. M. Brownlee, ed. Garland STPM Press, New York, NY.

21. Oscar, T. P., C. R. Baumrucker, and T. D. Etherton. 1986. Insulin binding to bovine mammary membranes: Comparison of microsomes versus smooth membranes. J. Anim. Sci. 62:179.

22. Posner, B. I., J. J. M. Bergeron, Z. Josefsberg, M. N. Khan, R. J. Khan, B. A. Patel, R. A. Sikstrom, and A. K. Verma. 1981. Polypeptide hormones: Intracellular receptors and internalization. Rec. Prog. Horm. Res. 37:539.

23. Roth, R. A. and D. Cassell. 1983. Insulin receptor: Evidence that it is a protein kinase. Science 219:299.

24. Seals, J. R., J. McDonald and L. Jarett. 1979. Insulin effects on protein phosphorylation of plasma membranes and mitochondria in a subcellular system from rat adipocytes. II. Characterization of insulin-sensitive phosphoproteins and conditions for observation of the insulin effect. J. Biol. Chem. 254:6997.

25. Vernon, R. G., E. Finley, E. Taylor, and D. J. Flint. 1985. Insulin binding and action on bovine adipocytes. Endocrinology 116:1195.

LIST OF REFERENCES

Banks, W., J. L. Clapperton, M. E. Ferrie, and A. G. Wilson. 1976. Effect of feeding fat to dairy cows receiving a fat-deficient basal diet. J. Dairy Res. 43:213.

Bar, R. S. and M. Boes. 1984. Distinct receptors for IGF-I, IGF-II, and insulin are present on bovine capillary endothelial cells and large vessel endothelial cells. Biochem. Biophys. Res. Commun. 124:203.

Bauman, D. E., D. L. Ingle, R. W. Mellenberger, and C. L. Davis. 1973. Factors affecting in vitro lipogenesis by bovine mammary tissue slices. J. Dairy Sci. 56:1520.

Bergman, E. M., R. P. Brockman, and C. F. Kaufman. 1974. Glucose metabolism in ruminants: Comparison of whole-body turnover with production by gut, liver, and kidneys. Fed. Proc. 33:1849.

Bergman, E. N., M. L. Katz, and C. F. Kaufman. 1970. Quantitative aspects of hepatic and portal glucose metabolism and turnover in sheep. Am. J. Physiol. 219:785.

Berlin, E., S. J. Bhathena, P. G. Kliman, and K. Revett. 1985. Effect of saturation of dietary lipids on insulin receptors and membrane fluidity in rabbits. Fed. Proc. 44:1236. (Abstr.)

Blei, A. T., D. C. Robbins, E. Drobny, G. Baumann, and A. H. Rubenstein. 1982. Insulin resistance and insulin receptors in hepatic cirrhosis. Gastroenterology 83:119.

Bolander, F. F., Jr., K. R. Nicholas, J. J. Van Wyk, and Y. J. Topper. 1981. Insulin is essential for accumulation of casein mRNA in mouse mammary epithelial cells. Proc. Natl. Acad. Sci. USA 78:5682.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248.

Brockman, R. P. 1978. Roles of glucagon and insulin in the regulation of metabolism in ruminants. A review. Can. Vet. J. 19:55.

Campbell, P. G. and C. R. Baumrucker. 1986. Characterization of insulin-like growth factor-1/ somatomedin C receptors in the bovine mammary gland. J. Dairy Sci. 69(Suppl. 1):163. (Abstr.).

Carrick, D. T. and N. J. Kuhn. 1978. Diurnal variation and response to food withdrawal of lactose synthesis in lactating rats. Biochem. J. 174:319.

Carrington, C. A., H. L. Hosick, I. A. Forsyth, and R. Dils. 1983. Milk-fat synthesis by lobules prepared from rabbit mammary gland: Response to insulin, corticosterone, prolactin and progesterone. J. Endocrin. 97:157.

Collier, R. J., D. E. Bauman, and R. L. Hays. 1977. Lactogenesis in explant culture of mammary tissue from pregnant cows. Endocrinology 100:1192.

Cuatrecasas, P. 1971. Insulin receptor interactions in adipose tissue cells: Direct measurement and properties. Proc. Natl. Acad. Sci. 68:1264.

Cuatrecasas, P. 1971. Properties of the insulin receptor of isolated fat cell membranes. J. Biol. Chem. 246:7265.

Cuatrecasas, P. 1971. Unmasking of insulin receptors in fat cells and fat cell membranes. J. Biol. Chem. 246:6532.

Cuatrecasas, P., B. Desbuquois, and F. Krug. 1971. Insulin-receptor interactions in liver cell membranes. Biochem. Biophys. Res. Commun. 44:333.

Cuatrecasas, P. and M. D. Hollenberg. 1976. Membrane receptors and hormone action. Pages 251-451 in Advances in protein chemistry. C. B. Anfinsen, J. T. Edsall, and F. M. Richards, eds. Academic Press, New York, NY.

Dawson, R. M. C. and P. Kemp. 1970. Biohydrogenation of dietary fats in ruminants. Pages 504-518 in Physiology of digestion and metabolism in the ruminant. A. T. Phillipson, ed. Oriel Press, Newcastle upon Tyne.

DeMeyts, P. A., A. R. Bianco, and J. Roth. 1976. Site - site interactions among insulin receptors. Characterization of the negative cooperativity. J. Biol. Chem. 251:1877.

Dolais-Kitabgi, J., F. Checler, J. Rey, O. Morin, and P. Freychet. 1981. Insulin stimulation of amino acid transport in primary cultured rat hepatocytes varies in direct proportion to insulin binding. FEBS Letters 128:321.

Dowben, R. M., J. R. Brunner, and D. E. Philpott. 1967. Studies on milk fat globule membranes. Biochim. Biophys. Acta 135:1.

Ehrenreich, J. R., J. J. M. Bergeron, C. P. Siekevitz, and G. E. Palade. 1978. Golgi fractions prepared from rat liver homogenates. I. Isolation procedure and morphological characterization. J. Cell Biol. 59:45.

Etherton, T. D. and O. A. Walker. 1982. Charcterization of insulin binding to isolated swine adipocytes. Endocrinology 110:1720.

Flint, D. J. 1982. Insulin binding to rat mammary gland at various stages of cell isolation and purification. Mol. Cell. Endocrinol. 26:281.

Flint, D. J. and D. W. West. 1983. Insulin receptors in milk. Br. Vet. J. 139:39.

Forsyth, I. A. 1983. The endocrinology of lactation. Pages 309-349 in Biochemistry of lactation. T. B. Mepham, ed. Elsevier Science Publishers, BV, New York, NY.

Freychet, P., R. Kahn, J. Roth, and D. M., Neville, Jr. 1972. Insulin interctions with liver plasma membranes: Independence of binding of the hormone and its degradation. J. Biol. Chem. 247:3953.

Freychet, P., J. Roth, and D. M. Neville, Jr. 1971. Insulin receptors in the liver: Specific binding of

[^{125}I] insulin to the plasma membrane and its relation to insulin bioactivity. Proc. Natl. Acad. Sci. USA 68:1833.

Freychet, P., J. Roth, and D. M. Neville, Jr. 1971. Monoiodoinsulin: Demonstration of its biological activity and binding to fat cells and liver membranes. Biochem. Biophys. Res. Comm. 43:400.

Froesch, E. R., C. Schmid, J. Schwander, and J. Zapf. 1985. Actions of insulin-like growth factors. Ann. Rev. Physiol. 47:443.

Gammeltoft, S. 1984. Insulin receptors: Binding kinetics and structure - function relationship of insulin. Physiol. Rev. 64:1321.

Ganong, W. F. 1975. Page 253 in Review of medical physiology. 7th ed. Lange Medical Publications, Los Altos, CA.

Gavin, J. R. III, P. Gorden, J. Roth, J. A. Archer, and D. N. Buell. 1973. Characteristics of the human lymphocyte insulin receptor. J. Biol. Chem. 248:2202.

Gill, R. D. and I. C. Hart. 1980. Properties of insulin and glucagon receptors on sheep hepatocytes: A comparison of hormone binding and plasma hormones and metabolites in lactating and non-lactating ewes. J. Endocrinol. 84:237.

Ginsberg, B. H., C. R. Kahn, and J. Roth. 1977. The insulin receptor of the turkey erythrocyte: Similarity to mammalian insulin receptors. Endocrinology 100:82.

Grizard, J. 1983. Insulin binding to skeletal muscle membranes in growing ruminating sheep fed different diets. Reprod. Nutr. Develop. 23:389.

Guyton, A. C. 1976. Page 1042 in Textbook of medical physiology. W. B. Saunders Co., Philadelphia, PA.

Hall, K. and V. R. Sara. 1983. Growth and somatomedins. Vit. Horm. 40:175.

Hart, I. C, J. A. Bines, S. V. Morant, and J. L. Ridley. 1978. Endocrine control of energy metabolism in the

cow: Comparison of the levels of hormones (prolactin, growth hormone, insulin and thyroxine) and metabolites in the plasma of high- and low-yielding cattle at various stages of lactation. J. Endocrin. 77:333.

Hartman, L., F. B. Shorland, and I. R. C. McDonald. 1954. Occurrence of trans-acids in animal fats. Nature 174:185.

Harvey, W. R. 1960. Least-squares analysis of data with unequal subclass numbers. USDA Res. Ser. ARS-20-80.

Haskell, J. F., E. Meezan, and D. J. Pillion. 1984. Identification and characterization of the insulin receptor of bovine retinal microvessels. Endocrionology 115:698.

Helminen, H. J. and J. L. E. Ericsson. 1970. Quantitation of lysosomal enzyme changes during enforced mammary gland involution. Exper. Cell Res. 60:419.

Helminen, H. J. and J. L. E. Ericsson. 1971. Effects of enforced milk stasis on mammary gland epithelium, with special reference to changes in lysosomes and lysosomal enzymes. Exper. Cell Res. 68:411.

Henriquez, D. S., H. M. Tepperman, and J. Tepperman. 1979. Effects of high-glucose and high-fat diets on concanavalin A binding to rat liver plasma membranes and on the amount and pattern of their glycoprotein carbohydrates. J. Lipid Res. 20:624.

Hintz, R. L., A. V. Thorsson, G. Enberg. and K. Hall. 1984. IGF-II binding on human lymphoid cells: Demonstration of a common high affinity receptor for insulin-like peptides. Biochem. Biophys. Res. Commun. 118:774.

Horino, M., L. J. Machlin, F. Hertelendy, and D. M. Kipnis. 1968. Effect of short-chain fatty acids on plasma insulin in ruminant and nonruminant species. Endocrinology 83:118.

Hove, K. 1978. Effects of hyperinsulinemia on lactose secretion and glucose uptake by the goat mammary gland. Acta Physiol. Scand. 104:422.

Hove, K. 1978. Maintenance of lactose secretion during acute insulin deficiency in lactating goats. Acta Physiol. Scand. 103:173.

Inagaki, Y. and K. Kohmoto. 1981. A method for the iodination of insulin and its binding to dissociated mouse mammary cells. Endocrinol. Japon. 28:29.

Inagaki, Y. and K. Kohmoto. 1982. Changes in Scatchard plots for insulin binding to mammary epithelial cells from cycling, pregnant, and lactating mice. Endocrinology 110:176.

Ip, C., H. M. Tepperman, J. DeWitt, and J. Tepperman. 1976. The effect of diet fat on rat adipocyte glucose transport. Horm. Metab. Res. 9:218.

Ip, C., H. M. Tepperman, P. Holohan, and J. Tepperman. 1976. Insulin binding and insulin response of adipocytes from rats adapted to fat feeding. J. Lipid Res. 17:588.

Jacobs, S., K. J. Chang, and P. Cuatrecasas. 1975. Estimation of hormone receptor affinity by competitive displacement of labeled ligand: Effect of concentration of receptor and labeled ligand. Biochem. Biophys. Res. Commun. 66:687.

Jacobs, S., E. Hazum, and P. Cuatrecasas. 1980. Digestion of insulin receptors with proteolytic and glycosidic enzymes - Effects on purified and membrane-associated receptor subunits. Biochem. Biophys. Res. Commun. 94:1066.

Jarett, L., J. B. Schweitzer, and R. M. Smith. 1980. Insulin receptors: Differences in structural organization on adipocyte and liver plasma membranes. Science 210:1127.

Jarrett, I. G., O. H. Filsell, and F. J. Ballard. 1974. Metabolic and endocrine interrelationships in normal and diabetic sheep. Horm. Metab. Res. Supp. Series 4:111.

Jones, R. G., V. Ilic, and D. H. Williamson. 1984. Physiological significance of altered insulin metabolism in the conscious rat during lactation. Biochem. J. 220:455.

Kahn, C. R. 1976. Membrane receptors for hormones and neurotransmitters. J. Cell Biol. 70:261.

Kahn, C. R. 1975. Membrane receptors for polypeptide hormones. Pages 81-146 in Methods in membrane biology. E. D. Korn, ed. Plenum Publishing, New York, NY.

Kahn, C. R., P. Freychet, J. Roth, and D. M. Neville, Jr. 1974. Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. J. Biol. Chem. 249:2249.

Kahn, C. R., I. D. Goldfine, D. M. Neville, Jr., and P. DeMeyts. 1978. Alterations in insulin binding induced by changes in vivo in the levels of glucocorticoids and growth hormone. Endocrinology 103:1054.

Keenan, T. W., D. J. Morre, D. E. Olson, W. N. Yunghans, and S. Patton. 1970. Biochemical and morphological comparison of plasma membrane and milk fat globule membrane from bovine mammary gland. J. Cell Biol. 44:80.

Kelly, P. A., B. I. Posner, T. Tsushima, and H. G. Friesen. 1974. Studies of insulin, growth hormone, and prolactin binding: Ontogenesis, effects of sex and pregnancy. Endocrinology 95:532.

King, G. L. and S. M. Johnson. 1985. Receptor-mediated transport of insulin across endothelial cells. Science 227:1583.

Kono, T. 1972. The insulin receptor of fat cells: The relationship between the binding and physiological effects of insulin. Pages 171-203 in Insulin action. I. B. Fritz, ed. Academic Press, New York, NY.

Kono, T. and F. W. Barham. 1971. The relationship between the insulin-binding capacity of fat cells and the cellular response to insulin. Studies with intact and trypsin-treated fat cells. J. Biol. Chem. 246:6210.

Krupp, M. and M. D. Lane. 1981. On the mechanism of ligand-induced down-regulation of insulin receptor level in the liver cell. J. Biol. Chem. 256:1689.

Kulski, J. K. and P. E. Hartman. 1983. Milk insulin, GH and TSH: Relationship to changes in milk lactose, glucose and protein during lactogenesis in women. Endocrin. Exper. 17:317.

Kulski, J. K., K. R. Nicholas, Y. J. Topper, and P. Qasba. 1983. Essentiality of insulin and prolactin for accumulation of rat casein mRNAs. Biochem. Biophys. Res. Commun. 116:994.

Lavau, M., S. K. Fried, C. Susini, and P. Freychet. 1979. Mechanisms of insulin resistance in adipocytes of rats fed a high-fat diet. J. Lipid Res. 20:8.

LeBlanc, J., A. Nadeau, M. Boulay, and S. Rousseau-Migneron. 1979. Effects of physical training and adiposity on glucose metabolism and ^{125}I-insulin binding. J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 46:235.

LeMarchand, Y., E. G. Loten, F. Assismacoponlos-Jeannet, M. Forgue, P. Freychet, and B. Jeanrenaud. 1977. Effects of fasting and streptozotocin in the obese-hyperglycemic (ob/ob) mouse. Diabetes 26:582.

Lindsay, D. B. 1978. Glucogenesis in ruminants. Biochem. Soc. Trans. 6:1152.

Lobitz, C. J. and M. C. Neville. 1977. Control of amino acid transport in the mammary gland of the preganant mouse. J. Supramol. Struct. 6:355.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

MacLeod, G. K., Y. Yu, and L. R. Schaeffer. 1977. Feeding value of protected animal tallow for high yielding dairy cows. J. Dairy Sci. 60:726.

Marinetti, G. V., L. Schlatz, and K. Reilly. 1972. Hormone-membrane interactions. Pages 207-276 in Insulin action. I. B. Fritz, ed. Academic Press, New York, NY.

Martin, R. J. and R. L. Baldwin. 1971. Effects of insulin and anti-insulin serum treatments on levels of metabolites in rat mammary glands. Endocrinology 88:868.

Marx, G. D. and E. V. Carnolo. 1963. Method of biopsy of the mammary gland of cows and goats. J. Dairy Sci. 46:576.

Mather, I. H. and T. W. Keenan. 1983. Function of endomembranes and the cell surface in the secretion of organic milk constituents. Pages 232-283 in Biochemistry of lactation. T. B. Mepham, ed. Elsevier Science Publishers, BV, New York, NY.

McAtee, J. W. and A. Trenkle. 1971. Metabolic regulation of plasma insulin levels in cattle. J. Anim. Sci. 33:438.

McElduff, A. and C. J. Eastman. 1981. The erythrocyte insulin receptor. Aust. J. Exper. Biol. Med. Sci. 59:439.

Meserole, V. K. and T. D. Etherton. 1984. Insulin binding to liver microsomes from lean and obese swine during growth to market weight. J. Anim. Sci. 59:650.

Metzler, C. M., G. L. Elfring, and A. J. McEwen. 1974. A package of computer programs for pharmacokinetic modeling. Biometrics 30:562.

Muggeo, M., R. S. Bar, J. Roth, C. R. Kahn, and P. Gorden. 1979. The insulin resistance of acromegaly: Evidence for two alterations in the insulin receptor on circulating monocytes. 48:17.

National Research Council. 1978. Nutrient requirements of dairy cattle. Natl. Acad. Sci. Washington, D. C.

Neter, J. and W. Wasserman. 1974. Pages 382-386 in Applied linear statistical models. R. D. Irwin, Inc., Homewood, IL.

Nicholas, K. R. and Y. J. Topper. 1983. Anti-insulin receptor serum mimics the developmental role of insulin in mouse mammary explants. Biochem. Biophys. Res. Commun. 111:988.

Nowak, J. and L. Dzialoszynski. 1967. Effect of experimental alloxan diabetes on the secretion and composition of goat milk. Acta Physiol. Pol. 18:595.

O'Keefe, E. and P. Cuatrecasas. 1974. Insulin receptors in murine mammary cells: Comparison in pregnant and nonpregnant animals. Biochim. Biophys. Acta 343:64.

Olefsky, J. M. 1976. Decreased insulin binding to adipocytes and circulating monocytes from obese subjects. J. Clin. Invest. 57:1165.

Olefsky, J. M. 1975. Effect of dexamethasone on insulin binding, glucose transport, and glucose oxidation of isolated rat adipocytes. J. Clin. Invest. 56:1499.

Olefsky, J. M. 1976. Effects of fasting on insulin binding, glucose transport, and glucose oxidation in isolated rat adipocytes. J. Clin. Invest. 58:1450.

Olefsky, J. M. and T. P. Ciaraldi. 1981. The insulin receptor: Basic characteristics and its role in insulin resistant states. Pages 73-116 in Diabetes mellitus. M. Brownlee, ed. Garland STPM Press, New York, NY.

Olefsky, J. M. and G. M. Reaven. 1975. Effects of age and obesity on insulin binding to isolated adipocytes. Endocrinology 96:1486.

Olefsky, J. and G. M. Reaven. 1974. The human lymphocyte: A model for the study of insulin-receptor interaction. J. Clin. Endocrinol. Metab. 38:554.

Olefsky, J. M. and M. Saekow. 1978. The effects of dietary carbohydrate content on insulin binding and glucose metabolism by isolated rat adipocytes. Endocrinology 103:2252.

Oscar, T. P., C. R. Baumrucker, and T. D. Etherton. 1986. Insulin binding to bovine mammary membranes: Comparison of microsomes versus smooth membranes. J. Anim. Sci. 62:179.

Pagano, G., M. Cassander, A. Diana, E. Pisu, C. Bozzo, F. Ferrero, and G. Lenti. 1981. Insulin resistance in the aged: The role of the peripheral insulin receptors. Metabolism 30:46.

Palmquist, D. L. and H. R. Conrad. 1978. High fat rations for dairy cows. Effects on feed intake, milk

and fat production, and plasma metabolites. J. Dairy Sci. 61:890.

Palmquist, D. L. and E. A. Moser. 1981. Dietary fat effects on blood insulin, glucose utilization, and milk protein content of lactating cows. J. Dairy Sci. 64:1664.

Peacock, M. L., R. S. Bar, and J. Goldsmith. 1982. Interactions of insulin with bovine endothelium. Metabolism 31:52.

Pitelka, D. R. and S. T. Hamamoto. 1983. Ultrastructure of the mammary secretory cell. Pages 29-70 in Biochemistry of lactation. T. B. Mepham, ed. Elsevier Science Publishers, BV, New York, NY.

Pocius, P. A., J. M. Dreels, J. E. Devery-Pocius, and C. R. Baumrucker. 1984. Techniques for preparation of bovine mammary smooth membranes. J. Dairy Sci. 67:2055.

Pollet, R. J. and G. S. Levey. 1980. Principles of membrane receptor physiology and their application to clincial medicine. J. Cardiovascular Med. 92:663.

Polychronakos, C., H. J. Guyda, and B. I. Posner. 1983. Receptors for the insulin-like growth factors on human erythrocytes. J. Clin. Endocrinol. Metab. 57:436.

Posner, B. I., J. J. M. Bergeron, Z. Josefsberg, M. N. Khan, R. J. Khan, B. A. Patel, R. A. Sikstrom, and A. K. Verma. 1981. Polypeptide hormones: Intracellular receptors and internalization. Rec. Prog. Horm. Res. 37:539.

Posner, B. I., P. A. Kelly, R. P. C. Shiu, and H. G. Friesen. 1974. Studies of insulin, growth hormone and prolactin binding: Tissue distribution, species variation and characterization. Endocrinology 95:521.

Rechler, M. M. and S. P. Nissley. 1985. The nature and regulation of the receptors for insulin-like growth factors. Ann. Rev. Physiol. 47:425.

Rechler, M. M., J. Zapf, S. P. Nissley, E. R. Froesch, A. C. Moses, J. M. Podskalny, E. E. Schilling, and R. E.

Humbel. 1980. Interactions of the insulin-like growth factors I and II and multiplication-stimulating activity with receptors and serum carrier proteins. Endocrinology 107:1451.

Rosen, P., B. Ehrich, E. Junger, H. J. Bubenzer, and L. Kuhn. 1979. Binding and degradation of insulin by plasma membranes from bovine liver isolated by large scale preparation. Biochim. Biophys. Acta 587:593.

Roth, R. A. and D. Cassell. 1983. Insulin receptor: Evidence that it is a protein kinase. Science 219:299.

Rutter, W. J., R. L. Pictet, and P. W. Morris. 1973. Toward molecular mechanisms of developmental processes. Pages 601-646 in Annual review of biochemistry. E. E. Snell, P. D. Boyer, A. Meister, and C. C. Richardson, eds. Annual Reviews, Inc., Palo Alto, CA.

Scatchard, G. 1949. The attraction of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. 51:660.

Schacterle, G. R. and R. L. Pollack. 1973. A simplified method for the quantitative assay of small amounts of protein in biologic material. Anal. Bioch. 51:654.

Seals, J. R., J. McDonald, and L. Jarett. 1979. Insulin effects on protein phosphorylation of plasma membranes and mitochondria in a subcellular system from rat adipocytes. II. Characterization of insulin-sensitive phosphoproteins and conditions for observation of the insulin effect. J. Biol. Chem. 254:6997.

Shorland, F. B. 1953. Animal fats: Recent researches in the Fats Research Laboratory, D.S.I.R., New Zealand. J. Sci. Food Agric. 4:497.

Smith, D. H., D. L. Palmquist, and F. L. Schanbacher. 1986. Characterization of insulin binding to bovine liver and mammary microsomes. Comp. Biochem. Physiol. In Press.

Steel, R. G. D. and J. H. Torrie. 1980. Pages 187-188 in Principles and procedures of statistics. McGraw-Hill, Inc., New York, NY.

Storry, J. E., P. E. Brumby, A. J. Hall, and V. W. Johnson. 1974. Response of the lactating cow to different methods of incorporating casein and coconut oil in the diet. J. Dairy Sci. 57:61.

Strong, C. R., I. Forsyth, and R. Dils. 1972. The effects of hormones on milk-fat synthesis in mammary explants from pseudopregnant rabbits. Biochem. J. 128:509.

Sun, J. V., H. M. Tepperman, and J. Tepperman. 1977. A comparison of insulin binding by liver plasma membranes of rats fed a high glucose diet or a high fat diet. J. Lipid Res. 18:533.

Taylor, S. I. 1975. Binding of hormones to receptors. An alternative explanation of nonlinear Scatchard plots. Biochemistry 14:2357.

Tepperman, H. M., R. Silver, J. DeWitt, and J. Tepperman. 1981. Effects of high glucose and high lard diets on the activities of rat liver glycosyltransferases. J. Nutr. 111:1734.

Terry, P. M., M. R. Banerjee, and R. M. Lui. 1977. Hormone-inducible casein messenger RNA in a serum-free organ culture of whole mammary gland. Proc. Natl. Acad. Sci. USA 74:2441.

Threadgold, L. C. and N. J. Kuhn. 1984. Monosaccharide transport in the mammary gland of the intact lactating rat. Biochem. J. 218:213.

Topper, Y. J. and C. S. Freeman. 1980. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev. 60:1049.

Trenkle, A. 1972. Radioimmunoassay of plasma hormones: Review of plasma insulin in ruminants. J. Dairy Sci. 55:1200.

Vernon, R. G., E. Finley, E. Taylor, and D. J. Flint. 1985. Insulin binding and action on bovine adipocytes. Endocrinology 116:1195.

Wahle, K. W. J. 1983. Fatty acid modification and membrane lipids. Proc. Nutr. Soc. 42:273.

Wiman, K., B. Curman, L. Tragardh, and P. A. Peterson. 1979. Demonstration of HLA-DR-like antigens on milk fat globule membranes. Eur. J. Immunol. 9:190.

Yalow, R. S. and S. A. Berson. 1960. Immunoassay of endogenous plasma insulin in man. J. Clin. Invest. 39:1157.

Zick, Y., N. Sasaki, R. W. Rees-Jones, G. Grunberger, S. P. Nissley, and M. M. Rechler. 1984. Insulin-like growth factor-I (IGF-I) stimulates tyrosine kinase activity in purified receptors from a rat liver cell line. Biochem. Biophys. Res. Commun. 119:6.