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Iodoprotein Formation by Rat Mammary Glands during Pregnancy and Early Postpartum Period (42279)

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Abstract. Iodide organification by rat mammary glands was studied during the trimesters of pregnancy and early postpartum period. Organification was followed by measuring trichloroacetic acid (TCA) precipitation of delipidated tissue homogenates. The radiolabeled material was sensitive to proteolytic cleavage by a bacterial protease indicating that the ^{125}I was protein-bound. Gel filtration column chromatography in the presence of sodium dodecyl sulfate (SDS) of delipidated mammary tissue homogenates of pregnant and postpartum rats reproducibly resolved several iodoproteins from free iodide. The K_{av} value for each iodoprotein peak was calculated and was used to estimate each subunit molecular weight which averaged 37,500, 25,100, and 8500. Another iodoprotein with a very large subunit molecular weight of greater than 300,000 was also detected in mammary tissue. Incorporation of ^{125}I-iodide into the three smaller iodoproteins increased logarithmically from the start of the second trimester of pregnancy through the early postpartum period when approximately 20% of the total ^{125}I uptake by mammary tissue was incorporated into protein. Hyperplasia, acinar development, and intracytoplasmic vacuolization of mammary tissue correlated with the increased incorporation of ^{125}I-iodide into these iodoproteins. The characterization and quantitation of specific iodoproteins in mammary tissue may be important as organification of iodide is believed to be a marker for normal hormone-responsive cells.

The glandular preparation for lactation begins early in pregnancy. During pregnancy ductal and acinar cells of mammary tissue are hormonally stimulated to divide, resulting in marked alveolar cellular hyperplasia (1). Concomitant to increased cell numbers are alterations in protein and lipid metabolism (2, 3) and changes in the ratio of RNA to DNA (4). Hormonal stimulation of rat mammary glands during pregnancy also results in the elaboration of a mechanism responsible for enhanced iodide uptake (5) and its organification (6). The organic binding of iodide during late pregnancy and lactation has recently been localized to protein-containing vacuoles in alveolar cells and to casein-like proteins in milk (7). The ability to organify iodide is correlated with mammary peroxidase activity in alveolar cells (7) and, at least in uterus, peroxidase activity is the result of estrogen-mediated gene regulation (8, 9).

Iodide has been found to be metabolically important in organs other than mammary glands including thyroid, salivary glands, stomach, and other secretory glands (10, 11). The importance of iodide metabolism in the normal maturation process of mammary glands has been suggested (12, 13). In rodents, iodide deficiency, either through dietary means or by perchlorate blockade, results in tissue changes similar histologically to human breast fibrocystic disease, dysplasia, and even neoplasia (14–16). The connection between enhanced iodide metabolism seen in pregnancy and the role of iodide in normal breast maturation in humans is not known; nor is the exact nature of the specific iodoproteins synthesized. A recent report suggests that resting human breast has the ability to iodinate protein which is related to the influence of hormones during the menstrual cycle (17). Radiolabeled secretory material consistent histologically with protein was observed in epithelial terminal ductules and intralobular

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ducts. The characterization of these iodoproteins may be important as the ability to organify iodide through biologic peroxidation is believed to be related to hormone responsiveness of cells (18, 19). Thus, iodoprotein production corresponds to peroxidase activity which in turn may be a specific marker for hormone dependence of normal mammary glands and also of mammary tumor cells (18, 20).

Materials and Methods. Thirty-four Sprague–Dawley virgin female rats (200 g) were housed three per cage and were given water and food *ad libitum*. The environment was controlled with a 12-hr light cycle and 12-hr dark cycle at 24°C. Nonpregnant female controls were separated and remaining rats were mated (three females to one male per cage). Pregnancy was confirmed by presence of sperm in vaginal smears (Day 0). Female rats were divided into five groups as follows: nonpregnant rats (not mated); 1 to 6 days pregnant; 7 to 13 days pregnant; 14 to 20 days pregnant; and 1 to 3 days postpartum. Term was calculated as 21 days after positive vaginal smears. Using this system, rat gestation was divided into trimesters. Progression of pregnancy was followed by monitoring maternal body weight. After delivery, litters were separated from their mothers before nursing began. Rats were sacrificed at the midpoint of the trimester and age of gestation was confirmed at necropsy using fetal crown–rump lengths and body weights. These measurements correlated well with the dates calculated from positive vaginal smears. At designated times, rats were injected via tail vein with 100 μCi of $Na^{125}I$ (New England Nuclear). Two hours later the rats were killed using ether, and mammary glands were dissected bilaterally and weighed. The right posterior mammary glands of each rat were used to assay ^{125}I radioactivity uptake while the left were used for biochemical analysis. Samples of mammary glands from both sides were examined histologically. ^{125}I uptakes of thyroid and mammary glands were determined by direct radioassay.

Fractionation of ^{125}I-iodinated compounds in tissues. Samples of mammary and thyroid tissue were quickly homogenized in saline using a Polytron (Brinkman Instruments, Westbury, N.Y.). One milliliter of the homogenate was then delipidated by mixing with 14 ml of cold chloroform/methanol 1/1, v/v. The mixture was incubated for 30 min at 4°C and then 14 ml of cold diethyl ether was added. After mixing the sample was incubated at 4°C overnight. Samples were then centrifuged at 2500 rpm for 15 min at 4°C in a Beckman L8-80 ultracentrifuge using a SW27 rotor. Lipids were removed by decanting the organic phase and delipidated tissue was air-dried to remove diethyl ether. Protein was precipitated from delipidated homogenates by two washes in hot 10%, w/v, trichloroacetic acid (TCA), and free ^{125}I-iodide was removed by removing the supernatants. The pellet was then radioassayed to determine radioactivity incorporated into ^{125}I-iodoprotein.

Sodium dodecyl sulfate (SDS)–gel filtration column chromatography of radiolabeled iodoproteins. Delipidated and TCA-precipitated protein from representative gestational groups was dissolved in MD buffer containing 10%, w/v, sodium dodecyl sulfate, 10%, v/v, glycerol, 10%, v/v, 2-mercaptoethanol in 0.1 *M* tris(hydroxymethyl) aminomethane buffer, pH 7.4. Dissolved samples were boiled for 2–5 min and then chromatographed on 1.5 × 170-cm columns containing Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, N.J.) in 0.01 *M* phosphate buffer containing 1%, w/v, SDS at an average flow rate of 7–8 ml/hr as described in detail by Sparks and Marsh (21). Fractions were collected every 30 min and aliquots were radioassayed. For proteolytic digestions, TCA-precipitated protein was digested with Pronase, a nonspecific protease isolated from *Streptomyces griseus* (Protease Type XIV-Pronase E, Sigma Chemical Co., St. Louis, Mo.). Precipitates were neutralized with NaOH, dissolved in 0.1 *M* borate buffer, pH 7.0, and digested overnight at 37°C. After incubation the mixture was mixed with an equal volume of MD buffer, boiled, and chromatographed as described above.

Histologic evaluation of mammary glands. Mammary glands were examined by routine hematoxylin and eosin staining procedures and histology was correlated with cellular modifications during pregnancy and with the extent of iodide organification in each trimester and in the postpartum period.

Radioactivity measurements. Radioassays of ^{125}I-labeled material of tissue homogenates,

TCA precipitates, or column fractions were made in a Searle 1144 γ scintillation spectrometer having a counting efficiency of 82%. Counting errors in all cases were kept less than 5%.

Results. *Histology.* Mammary glands of nonpregnant rats showed the normal histologic pattern of inactive tissue with abundant adipose stroma containing duct clusters with no evidence of lobular growth. In the first trimester of pregnancy (1 to 6 days pregnant) there was some duct proliferation but only minimal lobular change and altered parenchyma-to-fat ratio. In the second trimester of pregnancy (7 to 13 days pregnant) there was marked hyperplasia of the lobularacinar elements but without secretory activity. Hyperplasia was at a maximum in the early phase of the third trimester (14 to 20 days pregnant) when the ducts became relatively inconspicuous. Cytoplasmic vacuolization and luminal droplets became abundant and just prior to full term the pattern appeared like a honeycomb due to lobular secretory distention. The postpartum period was characterized by a secretory pattern of ductal epithelium. There was no evidence of metaplasia, atypia, or dysplastic changes in any microscopic sections examined in this study.

Uptake of ^{125}I by thyroid and mammary glands. The uptake and organification of injected ^{125}I-iodide by thyroid and mammary glands of control rats and postpartum rats were analyzed as described under Materials and Methods and results are presented in Table I. As seen in the table, the thyroid glands of control rats removed approximately 4% of the radioactivity injected and the majority of that taken up was used for the biological iodination of large subunit molecular weight proteins. SDS–gel filtration column chromatography of this product indicated its minimum subunit molecular weight to be on the order of 300,000, which is consistent with the known subunit molecular weight of thyroglobulin (330,000). In contrast to iodide uptake by thyroid gland, less than 0.3% of the radioactivity injected was removed by mammary glands of control rats, and its organification was minimal. The uptake of iodide by thyroid glands of postpartum rats was similar to that observed in control rats. In contrast to control rat mammary glands, the uptake of iodide by mammary glands of postpartum rats was significant and approximately one-fifth of the iodide removed was organified. Virtually all of that organified was incorporated into small subunit molecular weight proteins (<50,000).

Unlike thyroid uptakes which remained relatively constant throughout pregnancy, iodide uptake and protein organification by rat mammary glands increased progressively with duration of pregnancy. The increase in iodoprotein radioactivity of mammary glands of pregnant rats became statistically significant over nonpregnant controls ($P < 0.05$) during the second trimester and iodoprotein radioactivity was found to be logarithmically cor-

TABLE I. IODIDE UPTAKE AND ORGANIFICATION BY THYROID AND MAMMARY GLAND OF CONTROL AND POSTPARTUM RATS[a]

	Control rats ($N = 4$)		Postpartum rats ($N = 4$)	
	Thyroid gland	Mammary gland	Thyroid gland	Mammary gland
Iodide uptake	4.1 ± 1.3	0.23 ± 0.03	5.1 ± 1.2	6.73 ± 1.50*
Organified iodide	3.8 ± 1.2[b]	0[c]	4.5 ± 1.0[b]	1.25 ± 0.39[b,*]

[a] Results are expressed as a percentage of the total injected ^{125}I-iodide radioactivity. The molecular size distribution of the tissue homogenate radioactivity was determined by SDS gel filtration column chromatography as described under Materials and Methods. Rats were sacrificed 120 min after intravenous injection of ^{125}I-iodide.

[b] Organified protein in the thyroid gland (>90%) migrated with a column mobility corresponding to a molecular size greater than 300,000. Organified protein in the mammary gland migrated as described in Fig. 2.

[c] Less than 0.005% of the injected dose.

* Indicates a significant difference at a probability level of 0.05 (control vs postpartum).

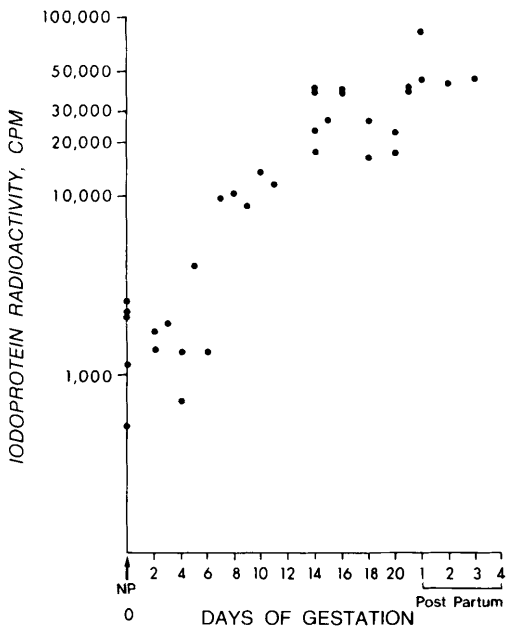

FIG. 1. The logarithmic relationship of ^{125}I-iodoprotein radioactivity with days of gestation. ^{125}I-Iodoprotein was assessed as the radioactivity of tissue homogenates remaining after complete delipidation and precipitation in 10%, w/v, trichloroacetic acid as described under Materials and Methods. The logarithm of ^{125}I-iodoprotein radioactivity of 0.25 g of tissue (cpm) was plotted against age of gestation.

related to gestational age as seen in Fig. 1 ($r = 0.976$, $P < 0.01$) and Table II.

Nature of the organified ^{125}I-iodide. SDS-gel filtration column chromatography of delipidated and TCA-precipitated mammary tissue homogenates was performed as described under Materials and Methods. As seen in Fig. 2, four iodoprotein peaks were reproducibly observed in mammary tissue homogenates from pregnant and postpartum rats. Iodoprotein radioactivity (peaks 2, 3, and 4) increased from the second trimester of pregnancy to the postpartum period. Iodoprotein radioactivity of peak 1 and free ^{125}I-iodide remained largely unchanged throughout gestation. Column analysis was performed on tissue from rats in each trimester of pregnancy and in the postpartum group and results gave similar radioactivity column distributions (data not shown). The protein nature of the radiolabeled material was confirmed by sensitivity to proteolytic cleavage. As seen in Fig. 3, iodoproteins from a delipidated and TCA-precipitated mammary tissue homogenate were chromatographed before and after Pronase treatment. All four iodoprotein peaks were removed by proteolysis and radioactivity was recovered as small peptides and amino acids (shaded area). No free ^{125}I-iodide was generated during the proteolytic treatment.

Subunit molecular weights of iodoproteins. The subunit molecular weights of the radiolabeled iodoproteins of mammary tissue were estimated by calculating the average fraction of the stationary gel volume available for diffusion for each iodoprotein (K_{av}). A standard curve was prepared by plotting the K_{av} of standard proteins against the logarithm of their molecular weights. The subunit molecular weights of iodoproteins 2, 3, and 4 were calculated to be 37,500, 25,100, and 8500, respectively. Fraction 1 cochromatographed with apolipoprotein B_H which suggested that its minimum subunit molecular weight was greater than 300,000. Both free ^{125}I-iodide and bromophenol blue had elution volumes greater than the total column volume giving K_{av}s greater than 1.0 indicating a matrix interaction with the agarose gel.

Discussion. The results presented here confirm previous reports that mammary glands from nonpregnant rats have limited ability to organify iodide (7, 23). The mammary glands of pregnant rats, however, have been shown

TABLE II. IODOPROTEIN FORMATION BY RAT MAMMARY GLAND DURING PREGNANCY AND EARLY POSTPARTUM PERIOD

Days of gestation	Number	Iodoprotein (cpm)
Nonpregnant	5	1,147 ± 216
1 to 6 days pregnant	7	2,221 ± 350
7 to 13 days pregnant	6	4,019 ± 1040
14 to 20 days pregnant	8	13,507 ± 1589
1 to 3 days postpartum	6	45,706 ± 891

Note. Protein organification was assessed as the radioactivity of 0.25 g of mammary gland homogenates which remained after complete delipidation and precipitation in 10%, w/v, trichloroacetic acid as described under Materials and Methods. Results are expressed as the mean values ± the standard error of the mean.

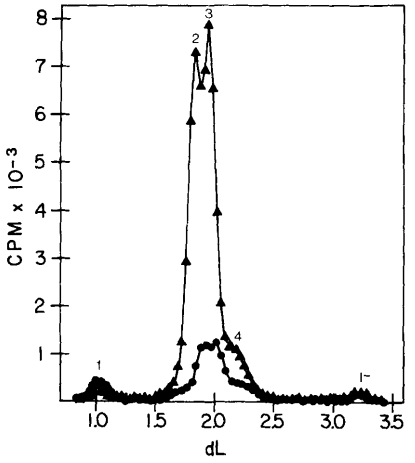

FIG. 2. SDS-gel filtration column chromatography of mammary tissue homogenates of pregnant (●) and postpartum rats (▲). Delipidated and TCA-precipitated mammary tissue homogenates of 18-day-pregnant and 1-day-postpartum rats were dissolved in MD buffer containing SDS as described under Materials and Methods. The samples were boiled and then chromatographed on 1.5 × 170-cm columns of Sepharose CL-6B at a flow rate of 7–8 ml/hr. Eluted fractions (3.7–3.8 ml) were radioassayed and cpm were plotted against the volume of elution in deciliters, dl. Four peaks of noniodide radioactivity were reproducibly seen in pregnant and postpartum rat mammary glands, designated as 1 through 4 in the figure. An additional peak, representing an insignificant fraction of the total radioactivity, was seen as free ^{125}I-iodide which is indicated on the figure as I^-. The subunit molecular weight of each iodoprotein peak was estimated by calculating the average fraction of the stationary gel volume available for diffusion (K_{av}). The void volume was taken as the volume of elution of Blue 2000 Dextran and the total column volume was the volume of elution of 2-mercaptoethanol. A standard curve was prepared by plotting the logarithm of the molecular weight of standard proteins against their calculated K_{av} values.

to acquire the ability to iodinate milk proteins during the last half of pregnancy (7, 13). The ability to organify iodide occurs shortly after the appearance of mammary peroxidase activity approximately on Day 12 (7). Our results are consistent with previous reports that there is a significant increase in organified iodide in the second trimester (7–13 days) and that the postpartum (lactating) mammary gland has marked ability to organify iodide (11, 13, 24).

The ability of rat mammary glands to iodinate proteins is believed to be related to hormonal stimulation by estrogen (19). Estrogen causes alveolar cell differentiation and probably induces the synthesis of endogeneous peroxidases as has been described in uterus studies (8, 9). This process occurs in the latter stages of pregnancy and is related to preparation for lactation. The significance of iodinated milk proteins to offspring is unclear although it may provide an avenue for absorption of the trace element, iodine.

Milk proteins, particularly tyrosine residues of caseins, are believed to be the substrate for alveolar cell mammary peroxidase although other enzymes in milk may have the ability to iodinate milk proteins (25). Mammary gland tissue slices can monoiodinate tyrosine residues of larger iodoproteins (22–24); however, the nature of the larger protein substrate has not been fully explored. In the current study mammary tissue homogenates were completely delipidated and proteins were precipitated by 10% TCA while TCA-soluble radioactivity was completely removed. Precipi-

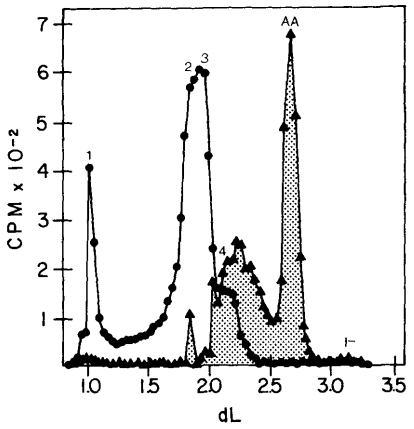

FIG. 3. SDS-gel filtration column chromatography of mammary tissue homogenates of pregnant rat before (●) and after (▲) proteolytic digestion. Split samples of delipidated and TCA-precipitated mammary tissue homogenates from 13-day-pregnant rats were digested with and without Pronase overnight at 37°C in 0.1 *M* borate buffer, pH 7.0, as described under Materials and Methods. After incubation the samples were mixed with an equal volume of MD buffer containing SDS and chromatographed as described in the legend to Fig. 2. The shaded area represents newly generated iodopeptides and iodotyrosines. The largest peak, with a volume of elution of approximately 2.7 deciliters (dl), marks the total column volume.

tated radioactivity was then solubilized and completely denatured in SDS. The protein nature of this radioactivity was confirmed by its susceptibility to a bacterial protease. The only protein found to be iodinated in thyroid glands using this procedure (results not shown) had a subunit molecular weight of approximately 300,000, consistent with that of thyroglobulin. In contrast to thyroid glands, three smaller iodoprotein subunits were found in pregnant and postpartum mammary tissue, and iodide incorporation into these proteins correlated logarithmically with gestation. Subunit molecular weights of the three smaller iodoproteins were found to average 37,500, 25,100, and 8500, respectively. Considering that caseins are believed to be substrates for mammary tissue peroxidase and that α- and β-caseins have subunit molecular weights of 26,000 and 25,000 (26), it is likely that peak 3 contains these iodinated milk proteins. The identity of the other iodoproteins of peaks 2 and 4 is not known. It is possible that the material in peak 4 may represent fragments of iodinated caseins. In this regard, it is interesting to note that renin acts on κ-caseins to release small glycopeptides of 6000 to 8000 molecular weight of variable TCA solubility. The exact identity of these iodoproteins will have to be confirmed immunologically.

In summary, rat mammary tissue develops the ability to organify iodide under the stimulation of pregnancy probably mediated through estrogen. Iodide organification correlates logarithmically with days of gestation and with the histological development of the ability of the mammary gland to secrete milk. Iodide is organified to proteins having average subunit molecular weights of 37,500, 25,100, and 8500. Iodoproteins having molecular weights of approximately 25,000 may represent iodinated α- and β-caseins. The identity of this and the other iodoproteins awaits further investigation. The characterization and quantification of specific iodoproteins in mammary tissue may be important as organification of iodide is believed to be a marker for normal hormone responsive cells (18, 20). Hormone-independent mammary tumors not only lose their hormone receptors, but also lose their ability to organify iodide (19, 20). Whether such tumors simultaneously lose the ability to iodinate one or more of the smaller iodoproteins found in this study remains to be investigated.

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1. Haagensen CD. The normal physiology of the breast. In: Haagensen CD, ed. Diseases of the Breast, 2nd ed., Philadelphia, Saunders, p62, 1971.
2. Salazar H, Tobon H, Josimovich JB. Developmental, gestational, and progestational modifications of the human breast. Clin Obstet Gynecol **18**:113–137, 1975.
3. Vorherr H. The Breast: Morphology, Physiology and Lactation, 2nd ed., New York, Academic Press, p107, 1967.
4. Masters JRW, Drife JO, Scarisbrick JJ. Cyclic variation of DNA synthesis in human breast epithelium. J Natl Cancer Inst **58**:1263–1265, 1977.
5. Maqsood M, Reineke EP. *In vitro* uptake of I^{131} by mammary tissue. Amer J Physiol **199**:829–832, 1960.
6. Brown-Grant K. The iodide concentrating mechanism of the mammary gland. J Physiol (London) **135**:644–654, 1957.
7. Strum JM. Site of iodination in rat mammary gland. Anat Rec **192**:235–244, 1978.
8. Lyttle CR, De Sombre ER. Uterine peroxidase as a marker for estrogen action. Proc Natl Acad Sci USA **74**:3162–3166, 1977.
9. Lyttle CR, Jellinck PH. Subcellular localization of estrogen-induced uterine peroxidase. Biochem J **160**: 237–241, 1976.
10. Honour AJ, Myant NB, Rowlands EN. Secretion of radioiodine in digestive juices and milk in man. Clin Sci **11**:447–462, 1952.
11. Brown J. Extrathyroidal iodide metabolism in the rat. Endocrinology **58**:68–78, 1956.
12. Eskin BA, Sparks CE, LaMont BI. The intracellular metabolism of iodine in carcinogenesis. Biol Trace Elem Res **1**:101–117, 1979.
13. Shah NM, Eskin BA, Krouse TB, Sparks CE. Iodide organification in the gravid rat breast. Proc Endocr Soc Aust **64**:410, 1982.
14. Aquino TI, Eskin BA. Rat breast structure in altered iodine metabolism. Arch Pathol **94**:280–285, 1972.
15. Eskin BA, Shuman R, Krouse T, Merion JA. Rat mammary gland atypia produced by iodine blockade with perchlorate. Cancer Res **35**:2332–2339, 1975.
16. Krouse TB, Eskin BA, Mobini J. Age-related changes resembling fibrocystic disease in iodine-blocked rat breasts. Arch Pathol Lab Med **103**:631–634, 1979.
17. Strum JM, Phelps PC, McAtee MM. Resting human female breast tissue produces iodinated proteins. J Ultrastruct Res **84**:130–139, 1983.
18. DeSombre ER, Anderson WA, Kang Y-H. Identification, subcellular localization and estrogen regulation

of peroxidase in 7,12-Dimethylbenz (a) anthracene-induced rat mammary tumors. Cancer Res **35**:172–179, 1975.

19. Strum JM. Autoradiographic evidence of a loss of iodination within hormone dependent GR mouse mammary tumors as they progress to independence. Anat Rec **204**:323–332, 1982.
20. Thorpe SM. Increased uptake of iodide by hormone-responsive compared to hormone-independent mammary tumors in GR mice. Int J Cancer **18**:345–350, 1976.
21. Sparks CE, Marsh JB. Analysis of lipoprotein apoproteins by SDS-gel filtration column chromatography. J Lipid Res **22**:514–518, 1981.
22. Potter GD, Tong W, Chaikoff IL. The metabolism of I^{131}-labeled iodine, thyroxine and triiodothyronine in the mammary gland of the lactating rat. J Biol Chem **234**:350–354, 1959.
23. Freinkel N, Ingbar SH. The metabolism of ^{131}I by surviving slices of rat mammary tissue. Endocrinology **58**:51–56, 1956.
24. Taurog A, Potter GD, Tong W, Chaikoff IL. The formation of I^{131} monoiodotyrosine from I^{131}-iodide by isolated particulate fractions of non-thyroid tissues. Endocrinology **58**:132–134, 1956.
25. Keston AS. The Schardinger enzyme in biological iodinations. J Biol Chem **153**:335–336, 1944.
26. McKenzie HA. Milk proteins. Adv Protein Chem **22**: 55–234, 1967.

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