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The Intracellular Distribution of Radioiodine Labeled Lactogenic Hormone in the Rabbit Mammary Gland

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INTRODUCTION

Investigations in the last three decades have provided a large body of material relative to lactational physiology and a somewhat smaller body of information has been obtained concerning the biochemistry of lactation. Because of these studies we have some insight into the control of mammary gland growth and secretory activity and a confusing and incomplete picture of the biochemistry of the mammary gland during these stages of growth and secretion.

Our knowledge of the physiology of the mammary gland may be said to date from the studies of Grueter (1928) and Stricker and Grueter (1928, 1929) on the effects of anterior pituitary extracts on the initiation of milk secretion. These studies showed for the first time that lactation was the result of a positive stimulus resulting in the activation of a specific tissue, the mammary gland, rather than a passive phenomenon occurring due to the completion of a growth phase with resulting maturation of the secretory cells. Studies of the role of the ovarian hormones in the physiology of mammary gland growth date from this same period and were begun in this laboratory by Turner and his associates and augmented by the work carried on in other laboratories.

Various studies using the techniques of biochemistry which were developed with other mammalian tissues, the liver and kidney in particular, have yielded information as to some of the metabolic and other biochemical changes which take place in the mammary gland as the physiological state of the gland is altered. These studies have not, however, given us the basic information which is sought; but have only shown that as the physiology of the mammary gland is altered, so must the biochemistry of the gland be altered.

We have not yet found the point at which the physiological control mechanisms impinge upon the biochemistry of the mammary gland cell. We have only observed the changes which are the result of the initial action of the controlling hormones on the mammary gland secretory cells. It is to this point which we must direct our efforts if a satisfactory understanding of the effect of a particular hormone upon a particular type of cell is to be obtained. The research which will be reported is, then, an attempt to elucidate the location and nature of the initial point of effect of the lactogenic hormone of the anterior pituitary on the secretory cells of the mammary gland.

REVIEW OF LITERATURE

Studies over the last 25 years have given us some insight into the physiological activity of the lactogenic hormone. These various activities have received attention in numerous reviews (Riddle, 1938; Turner, 1939; Folley and Malpress, 1948a, 1948b; Petersen, 1948; Folley, 1952). It will be of interest here, however, to inquire into those studies which have a direct bearing on the problem of the marked action of the lactogenic hormone upon the mammary gland metabolism.

The existence of a hormone controlling the initiation of lactation was first shown by Stricker and Gruter (1928) when they demonstrated that anterior pituitary extracts would initiate lactation in the pseudopregnant rabbit. Confirmation of this finding came from many groups, Corner (1930), Nelson and Pfiffner (1930, 1931), Turner and Gardner (1931), and Asdell (1931).

Changes in Gland Composition Reflecting Lactogenic Hormone Action

Weatherford (1929) found mitochondria present in the secretory cells of the mammary gland of the albino rat and observed them under various physiological conditions. Jeffers (1935) reported the appearance of cytoplasmic particulates at all stages of activity in the secretory cells of the mammary gland in rats. In the non-secreting cell these were granular or rod shaped and in fully active cells were in the form of filaments. Dempsey *et al.* (1947) examined the mammary glands of albino rats and found in the active alveolar cell, rods and filaments in the basal and nuclear regions of the cell.

In recent years biochemical studies have been made which show some striking differences between the lactating and non-lactating mammary gland cell. Jeffers (1935) reported that during pregnancy small fat droplets were observable throughout the cytoplasm; whereas, in the lactating cell, larger fat drops are accumulated at the luminal end of the cell. Studies of glycogen by Petersen and Shaw (1938) have revealed no differences in content between a lactating and a non-lactating cattle mammary gland.

Kleiber *et al.* (1943) reported on studies of rat mammary gland composition. They noted that the water and protein content of the gland increased with lactation. McShan *et al.* (1950), in studies of the effect of lactogenic hormone on the pigeon crop gland, report striking increases in pentose nucleic acid concentration. With five days' treatment the concentration was increased as much as 150 percent over the untreated controls. Smaller but significant increases were noted in desoxyribose nucleic acid, fresh tissue weight, and dry weight of the glands.

Kirkham and Turner (1953) have studied several components of the rat mammary gland cell in relation to growth and synthetic activity during

pregnancy and lactation. They reported that DNA increased during the first half of pregnancy and then remained essentially constant through lactation, indicating that the number of cells remained reasonably constant from the mid-point of pregnancy on. Their data showed increased amounts of PNA throughout the entire course of pregnancy and lactation. Of interest are the PNA/DNA ratio and the total nitrogen curves which they observed. Both showed an increase throughout pregnancy and lactation; however, the rate of increase appeared to be much greater in lactation than in pregnancy, indicating a higher PNA and nitrogen content per cell in the lactating gland, compared to the non-lactating condition during pregnancy.

Changes in Enzyme Activity Reflecting Lactogenic Hormone Action

Various enzymes of the mammary gland have been studied in relation to changes in gland physiology. Alkaline phosphatase was probably the first to be demonstrated in the mammary gland by Kay (1925). Folley and Kay (1935) reported that glands in active secretion show less phosphatase activity per gram than inactive glands. In view of later studies it appears that this finding may have been somewhat misleading due to the presence of milk in the lactating gland.

Folley and Greenbaum (1947) made a study of phosphatase of the mammary gland of the pregnant and lactating rat. They found that the level of phosphatase increased steadily throughout pregnancy and early lactation and maintained a maximal value throughout lactation.

Another hydrolytic enzyme, arginase, was reported by Shaw and Petersen (1938) to be present in the lactating mammary gland. Folley and Greenbaum (1947) showed that the great increase in arginase came through the period of lactation and was at a maximum at the end of lactation.

Some other single enzymes have been reported to be present in the mammary gland and to undergo change at the initiation of lactation. Greenstein and Jenrette (1941) reported the presence of a desoxyribonuclease in mouse mammary extracts which showed an elevated level in the lactating tissue.

McShan and his co-workers (1950) studied the changes in activity of an oxidative enzyme which were brought about by lactogenic hormone treatment. They report that with lactogenic hormone treatment the pigeon crop gland shows a striking increase in succinic dehydrogenase activity. Kocsis and Abood (1954) also noted marked increases in acid phosphatase activity of pigeon crop gland after four days' treatment with a lactogenic hormone preparation.

Moore and Nelson (1952) noted that the two oxidative enzymes which they studied in the mammary gland changed markedly between pregnancy

and lactation. In both the rabbit and guinea pig they found succinic dehydrogenase at low levels until the last third of pregnancy at which time a marked increase was found which reached and maintained a peak throughout lactation. With the enzyme cytochrome oxidase, the increase above the level during pregnancy apparently took place just at parturition and a peak was reached at about the height of lactation.

In a study of several of the enzymes which may play a role in protein synthesis, Greenbaum and Greenwood (1954) found several striking changes in activity in the rat mammary gland through pregnancy, lactation, and involution. Glutamic dehydrogenase was found to increase greatly the last half of pregnancy, fall sharply at the start of lactation, and then to increase to a peak by the fifteenth day of lactation and fall sharply with involution. Glutamic-aspartic transaminase and cathepsin were found to increase throughout the entire period of pregnancy and lactation. β-glucuronidase was found to increase to a peak at the end of pregnancy, fall sharply throughout lactation, and then increase again with the onset of involution.

Glock and McLean (1954) made the same type of study in the rat mammary gland in relation to oxidations of the hexose-monophosphate shunt. They found that the level of glucose-6-phosphate, 6-phosphogluconate dehydrogenase and the rate of breakdown of ribose-5-phosphate all increase from low levels at the end of pregnancy to peak values at the end of 21 days' lactation and fall sharply with the beginning of involution. Ringler, Becker, and Nelson (1954) report studies of mammary gland concentration of two co-enzymes at various stages of pregnancy and lactation. Co-enzyme A remained at a low level throughout pregnancy in the guinea pig but increased rapidly after parturition to a peak at the fifth or sixth day of lactation, after which the level gradually declined. The diphosphopyridine nucleotide (DPN) concentration was also studied through pregnancy and lactation and it was found that the concentration of DPN remained relatively constant throughout, although there may have been a small increase with lactation.

Changes in Metabolic Activity Reflecting Lactogenic Hormone Action

In one of the earliest papers studying the oxidative activity of mammary tissue, Kleiber *et al.,* (1943) noted an increase in the QO_2 of rat mammary tissue with lactation on a dry weight basis but a decrease on the basis of nitrogen. Other work by Knodt and Petersen (1946) indicated that the main pathway of oxidation of glucose was by oxidation to pyruvate.

Folley and French (1949a, 1949b, 1950) in metabolic studies of the rat mammary gland *in vitro* found that mammary gland slices from pregnant rats, in the presence of glucose, metabolized with a respiratory quotient (RQ) of less than one while slices from lactating gland showed an RQ greater than one, which confirms earlier work on the goat by Reineke *et al.*

(1941). Secondly they observed that the respiration rate of slices from lactating rats was higher than the respiration rate of slices from rats pregnant twenty days.

Balmain and Folley (1952) obtained interesting results pointing up the changes in metabolism which take place in the lactating gland, compared with the non-lactating gland. It was found in *in vitro* experiments on rat mammary slices that cortisone markedly affected the tissue from pregnant animals. Lactogen under the same conditions, was just the reverse: It had no effect on pregnant animal tissue and marked effects on lactating tissue.

In a study of oxygen consumption, metabolic carbon dioxide production, aerobic glycolysis, and RQ of rat mammary slices *in vitro,* Hoover and Turner (1954) found rather striking changes with the onset of lactation or the beginning of involution. They reported that the QO_2 and the carbon dioxide production increased gradually throughout pregnancy. With the onset of lactation they exhibited a striking increase, both reaching a peak at about the tenth day of lactation and remaining at high levels until the beginning of involuion. The apparent aerobic glycolysis showed little change through pregnancy and lactation but increased markedly with involution. The RQ was calculated to be less than one during pregnancy, to increase sharply to a maximum of 1.6 during lactation, and to fall with the onset of involution to values less than one.

Cellular Structure in Relation to Metabolism

This review of previous work has demonstrated that a protein hormone, lactogenic hormone (prolactin) of the anterior pituitary, acts directly upon the secretory cells of the mammary gland to initiate and maintain lactation. Work cited has shown that certain histological, biochemical, and metabolic changes take place in the mammary tissue as lactation is initiated and maintained. The question of how the lactogenic hormone brings about these changes in mammary tissue which results in lactation now becomes important. Where does the lactogenic hormone act? Where are the metabolic centers of the secretory cells where synthesis is taking place? How does the lactogenic hormone control and direct this activity? Some of these questions may be answered in part; much will have to be inferred from work carried out on other tissues and much more remains to be answered in the future.

If we turn to the excellent review by Green (1951) of the cyclophorase complex of enzymes, an insight into the general mechanism of the cell in certain aspects of its activity may be obtained. He defined the cyclophorase system as that complex of enzymes which catalyze the reactions of the citric acid cycle. It was found independently by Schneider and Potter (1949) and Kennedy and Lehninger (1949) that the mitochondria of the cell are the structural unit of this cyclophorase system of enzymes. These mitochondria, first isolated by Bensley and Hoerr (1934) have been investigated by

Chargaff (1942) and others (Bensley, 1937; Swanson and Artom, 1950) who have determined the chemical composition of the mitochondria and have found that they are made up in large part of ribonucleic acid, phospholipid, and protein and thus may be considered nucleoprotein in nature. This finding of metabolic function in the mitochondrial particulates of the cell is vastly important in that it provides known function for a portion of the cell long observed and studied by the histologist. Repeating some citations from Green (1951) on the types of reactions which have been identified with the mitochondria gives a picture of this structure's importance in the cellular scheme of things. He cites (1) the citric acid cycle of oxidation, (2) fatty acid oxidation, (3) some glycolytic enzymes, (4) transaminases, (5) oxidative phosphorlation mechanisms, and (6) the co-enzymes necessary for each of these systems. This is, undoubtedly, only a partial listing and other activities will be added in the future.

The microsomes, upon which considerably less study has been lavished, are a class of smaller cellular particulates with a size range of 50-150 mu. In gross chemical composition, the microsomes which have been studied, mammalian liver microsomes in particular, are somewhat similar to the mitochondria, the greatest difference being in a larger percent of lipid and ribonucleic acid (Dounce, 1950). A few enzyme studies have been made on the microsomes. Omachi *et al.* (1948) found esterase predominantly in the liver microsomes and Hogeboom (1949) found DPN-Cytochrome c reductase there also. Jeener (1948) has shown thrombokinase to be concentrated in the liver cell microsomes. De Meio *et al.* (1953)found that rat liver supernatant fractions which were able to synthesize phenyl sulfate were inhibited by the presence of rat liver microsomes, presumably because of the high ATP-ase and low sulfatase activity of the microsomes.

In mammary gland studies, Popjak and Tietz (1953), using carboxyl C^{14} labeled acetate, found that the highest level of synthesis of the acetate into fatty acids occurred in the homogenate fraction containing microsomes and soluble materials. Microsomes alone gave little activity. Sedimented microsomes, added back to the supernatant containing only soluble materials, increased activity but not to the extent noted before sedimentation. These findings with microsomes may have been due to changes during the sedimentation, i.e. loss of co-factors, or due to poor dispersion of the microsomes after being sedimented. At any rate, this work seems to implicate the microsome of the rat mammary gland as being involved in fatty acid synthesis.

At least one other important activity has been inferred for the microsomal particulates, that of a role in protein synthesis. Hultin (1950a) injected chicks with N^{15} labeled glycine and sacrificed the animals at different time intervals. The livers were homogenized and fractionated by differential

centrifugation and the N^{15} incorporated into the protein of the various fractions was determined. The time course of the incorporation showed that the amino acid was incorporated in the microsomes first, then into the cytoplasmic supernatant, and then into the mitochondria.

In vitro work by Borsook *et al.* (1948) with mitochondrial and microsomal fractions obtained from guinea pig liver homogenates gave interesting results in terms of amino acid utilization. C^{14} labeled glycine was taken up in microsomes to only 23 percent of the level attained in mitochondria; however, when the two particulate fractions were mixed the glycine was taken up to a level 254 percent more than that attained in the mitochondria alone. Runnstrom (1952) suggested that these results may be due to the increase in energy supply available for incorporation when the mitochondria are present.

Postulated Mode of Hormone Action

The discussion so far suggests that the mammary gland, under the influence of lactogenic hormone, exhibits some rather striking changes in histology, biochemical composition, and metabolism. Further, many of the enzyme systems important to the metabolism of the cell appear closely related to certain histological loci in the cell. There is some evidence suggesting that the various histological portions of the cell may be important in the action of hormones on the cellular level. This evidence will now be examined.

To quote from Baldwin (1949) concerning enzyme-substrate relationships, it may be possible to use this as a valuable and possibly valid insight into the manner in which hormones affect enzyme systems and thus exert varied effects upon the metabolism of a particular group of cells.

"It is difficult to imagine how a catalyst of any kind can influence the rate of a chemical reaction unless it actually participates in that reaction. Most authorities agree that catalysts do in some manner combine with the substance or substances upon which their catalytic influence is exerted, but there has been much difference of opinion as to whether the union is of a 'physical' or adsorption kind, or whether it is to be regarded as 'chemical.' But it is difficult to maintain that there is any fundamental difference between these types of union; rather must they be regarded as two extremes of one and the same phenomenon. . . . There is nothing inherently improbable in the idea that an enzyme actually unites with its substrate, and it is difficult indeed, to imagine how the facts of specificity could otherwise be accounted for."

It may also be possible to theorize along the above lines in regard to hormone action, since the same considerations must be accounted for in explaining activity in both cases. Thus Stadie *et al.* (1949a), in their studies of insulin action state: "Regardless of whether or not intact cellular morpho-

logy is an obligatory requirement for insulin action, the first step in the action of insulin in cellular systems must be its entrance into, or attachment to, some morphological element of the intact cell."

This concept has been discussed further by Szego and Roberts (1953) in the paper they presented for discussion at the ninth annual meeting of the Laurentian Hormone Conference:

"It has been generally assumed that the hormones exert their regulatory influences on growth and metabolism by modifying the activity of specific enzyme systems. The alternative possibility has been advanced, however, that the hormones, like certain drugs, may alter the permeability of the target cell by orientation at its surface. Each mechanism, or combination of the two, may be the basis for hormone action in different instances.

"The evidence in favor of a direct effect of the endocrine secretions on enzymatic activity stems mainly from *in vitro* experiments. Although it has frequently been possible to modify the activity of isolated metabolic systems, the relationship of these *in vitro* effects to physiological situations has rarely been demonstrated. The recognized capacity of the hormones to influence metabolism may be secondary to changes in cell permeability. It should be noted, however, that membrane permeability is a very complex phenomenon, subject to many physical, chemical, and biological influences. Hormonal alteration of the highly specific semipermeability of the cell membrane could be accomplished not only by orientation of the active molecules on the cell surface, but also by interaction with metabolic systems responsible for maintaining the state of the cell membrane. In any case, it appears that hormone action must depend upon relatively specific combination between these biocatalysts and certain cell constituents. The ability of the hormones to form strong links with protoplasmic components has been demonstrated in the case of insulin; which appears to attach itself firmly to the membrane of the muscle cell before exerting its characteristic effect on muscle glycogenesis. Similarly, some of the steroid hormones appear to exert a strong affinity for protein, and this combination may precede their action in the organism."

It would appear from this discussion that some association of the hormone with a component or components of the cell must be postulated, however transitory this may be. Certainly to date the locus of such an association in the cell has not been described, although evidence is being accumulated which may illuminate this point.

Evidence Supporting Postulated Mode of Action

For the sake of completeness it is necessary first to turn our attention to the field of immunology and note some of the more recent studies of antigen-antibody relationships which are in many ways similar to the present

problem of hormone action. There is one good review of these studies (Pressman, 1953) which illustrates the techniques which have been used in this field and the results obtained with them. Only the more recent work which has been accomplished with the aid of radioactive tracers will be discussed here.

It is of interest to note some findings by Haurowitz and his associates (Crampton and Haurowitz, 1950; Haurowitz *et al.*, 1951; and Crampton and Haurowitz, 1952) in this separate field of immunology. They have found that antigen proteins are bound into the cells of various organs and that the site of this binding may be localized in certain histological portions of the cell. This would appear to be a case of association of a specific protein with a specific portion of the cell to induce a specific biochemical change, i.e. antibody production.

In another study of antigen-antibody relationships Pressman (1951) found that antitissue antibodies for a particular tissue labeled with radioactive iodine (I^{131}) or radioactive sulfur (S^{35}) could be found to localize to a high degree in their specific organ. Thus he found that rat kidney tissue antibodies localized to a great extent in rat kidney tissue following injection into rats and to a lesser extent in liver. Two other interesting observations were made in this work; first, it was found that the half-life of the antikidney antibody was of the order of twenty days, and second, that the localization site was insoluble but that the labeled antibody could be eluted from this localization site by appropriate means (Eisen and Pressman, 1950; Pressman and Sherman, 1951). This finding of insolubility of the localization site is of interest in that it was resistant to saline from 0.16 M to 1.6 M and was resistant to acetone-ether extraction. The antibody could not be eluted with sodium chloride solutions over a range of concentration from 0.16 M. to 4.0 M. At low pH little material was eluted; however, in dilute alkali two-thirds or more of the activity was eluted from the insoluble portion of kidney homogenate sedimented at 2000 x g. for 30 minutes.

Returning now to hormone investigations, Stadie and his co-workers have studied insulin under a variety of conditions (Stadie *et al.*, 1949a; Stadie *et al.*, 1949b; Stadie, Haugaard, and Marsh, 1951; Hills and Stadie, 1952; Haugaard and Marsh, 1952). They have found that rat diaphragm muscle, rat adipose tissue, and rat lactating mammary gland all exhibit the ability to bind insulin in a stable combination at the time when a metabolic effect of insulin can be demonstrated in the tissue.

The earliest study of the distribution of a protein hormone using a radioactive tracer was that of Reiner, Keston and Green (1942) in which they coupled insulin with I^{131} tagged azoiodobenzene and obtained results suggesting the concentration of insulin in liver and kidney. Stadie, Haugaard, and Vaughan (1952), in a continuation of these studies of insulin binding of various tissues, used insulin isotopically labeled with S^{35} and I^{131}

to arrive at a measure of the insulin binding in rat diaphragm muscle. They report a binding, apparently chemical, of up to 1.5 micrograms insulin per gram of fresh tissue. From this it would appear that at least in the case of insulin the binding takes place in a reasonably stable chemical bonding and is a prerequisite for activity.

Lee and Williams (1954) in a recent study using I^{131} labeled insulin injected intravenously into rats to investigate liver uptake, report an active concentration of the insulin by liver tissue. With homogenate fractionation procedures, they report an almost equal distribution pattern between the liver cell mitochondria, microsomes and supernatant 5 minutes after injection. The pattern changes at 60 minutes so that more than half of the activity is found in the supernatant fraction. Thus, these studies show the entrance of a hormone into the cell and its active distribution intracellularly.

Gross and Schwartz (1951) studied the distribution of thyroxine labeled with radioactive iodine (I^{131}) in mice and noted a strong concentration of the thyroxine in liver, intestinal tract and kidney. Of the other organs studied the activity ranged downward in the following order, adrenal, skin, and mammary gland. All were above the level of concentration of the general carcass but they did not show a high degree of concentration. Gross and Pitt-Rivers (1954) have demonstrated recently that whereas thyroxine shows little concentration from plasma other than in the liver, triiodothyronine labeled with radioactive iodine (I^{131}) is taken up strongly by many organs. This seems to imply that triiodothyronine is the thyroid hormone which acts upon the cell.

Van Arsdel *et al.,* (1954) made a comparative study of the distribution of I^{131} labeled thyroxine and triiodothyronine and reported some interesting differences. Triiodothyronine concentration was higher in the adrenal gland, pancreas, heart, brain, kidney, large and small intestines, and muscle, while thyroxine concentration was higher in lung, stomach, testis and liver. Of great interest is the finding that the total muscle mass contained 10 percent of the injected thyroxine and 20 percent of the injected triiodothyronine during the first few hours after injection. Their data suggest a more rapid increase in triiodothyronine in muscle immediately following injection, followed by a period in which the rate of hormone turnover is similar. These findings seem to lend support to those of Gross and Pitt-Rivers (1954).

Several other studies have indicated that here again some degree of specific intracellular association may be requisite for activity. Lipner, Barker, and Winnick (1952) found thyroxine labeled with I^{131} and C^{14} distributed in rat liver homogenate fractions in a very close relationship to the dry weight of these fractions. This would appear to show that some active distribution of the thyroxine molecule was taking place in the liver cell. Carr and Riggs (1953) in a study of protein-bound-iodine in rat liver found that the PBI was distributed in homogenate fractions in the same relationship

as the protein of these fractions, substantiating the findings of Lipner, Barker, and Winnick (1952). Lee and Williams (1954) reported on the distribution in liver of thyroxine-I^{131} injected intravenously into rats. They found with homogenate fractionation procedures that at five minutes post-injection the great majority of activity in the liver cell was present in the nuclear and mitochondrial fractions. This binding did not appear to be a firm one in that large losses occurred with dialysis or perfusion. At 60 minutes post injection their findings look much like those of Lipner, Barker, and Winnick (1952).

Lardy and Maley (1954) report interesting *in vitro* results with dl-I^{131} thyroxine. They incubated a rat liver mitochondrial suspension with this labeled thyroxine and found that 80 percent of the added thyroxine was bound to the mitochondria after two washes with isotonic sucrose. The thyroxine was not displaced by washing with isotonic electrolyte solution nor by treatment with the reducing agent cysteine. This apparently represents a non-specific adsorption of thyroxine by mitochondria.

Sonenberg *et al.* (1952) report that studies with a thyrotrophic hormone preparation, known to contain large amounts of gonadotropic activity, which was coupled with diazobenzene sulfonic acid labeled with S^{35}, showed significant concentrations in the thyroid, gonads, and liver following intravenous injection into the male chick.

Other work of this nature has been carried out by Sonenberg and Keston and their associates with ACTH. Sonenberg, Keston, and Money (1951a, 1951b) studied the distribution of an ACTH preparation labeled with I^{131} and reported that their trace labeled product was actively taken up by the adrenal cortex. This work also suggested a specific binding between the gland constituents and the hormone preparation.

Sonenberg *et al.* (1951) in a study of localization of I^{131} trace labeled lactogenic hormone in the female rat claimed that they were not able to detect significant localization in the mammary gland. But they did find extensive activity bound to the ovaries, liver, spleen, kidney, and adrenals. They have no explanation for this lack of localization in the mammary gland, particularly in view of the findings of Cox (1951) that in the mouse appreciable amounts of I^{131} labeled lactogenic hormone are found in the mammary glands, adrenals, and ovaries 20 minutes after intravenous injection. It is well to note, however, that Sonenberg and his associates (1951) found a somewhat higher concentration of material in the mammary glands than in muscle. This might indicate some small degree of mammary localization.

In a review, Sonenberg (1952) reported his general findings from the use of anterior pituitary hormones labeled with I^{131}. With ACTH, concentration occurred in the rat adrenal. Trace-tagged growth hormone localized in the pancreas to a greater extent than a variety of other proteins or free

TISSUE LOCALIZATION OF RADIOACTIVELY TAGGED MATERIALS

tissue	acth	estrogens	growth	insulin	lactogenic	luteotropic	thyrotropic	thyroxine	antigens antibodies
adipose		1		6				23	
adrenal	14,17,18	1			2,15			5,23	
kidney		10		13	15			5,23	4,7,12
liver		1,10		13,9	15		16	5,9,11,23	3,4,7,12
lung								23	4,7,12
mammary		1,10		8	2			5	
muscle				19,20,21,22				23	4
ovary		1,10			2,14,15	14		23	
pancreas			14					23	
pituitary		1						23	
salivary		1							
skin		1						5	
spleen					15			23	4,7
thyroid							16		
uterus		1,10							
vagina		10							

REFERENCE NUMBERS

1. Albert et al. (1949)
2. Cox (1951)
3. Crampton and Haurowitz (1950)
4. Crampton and Haurowitz (1952)
5. Gross and Schwartz (1951)
6. Haugaard and Marsh (1952)
7. Haurowitz et al. (1951)
8. Hills and Stadie (1952)
9. Lee and Williams (1954)
10. Lewison et al. (1951)
11. Lipner, Barker and Winnick (1952)
12. Pressman (1951)
13. Reiner, Keston and Green (1942)
14. Sonenberg (1952)
15. Sonenberg et al. (1951)
16. Sonenberg et al. (1952)
17. Sonenberg, Keston and Money (1951a)
18. Sonenberg, Keston and Money (1951b)
19. Stadie et al. (1949a)
20. Stadie et al. (1949b)
21. Stadie, Haugaard and Marsh (1951)
22. Stadie, Haugaard and Vaughan (1952)
23. Van Arsdel, Hogness, Williams and Elgee (1954)

Figure 1

iodide. He reports that with both lactogenic hormone and luteotropic hormone there was significant localization in the ovaries. These latter two appear to be a reasonably firm combination in the ovary, since only a small loss occurred upon perfusion.

Few studies have been made in the area of hormone localization dealing with hormones which are neither proteins nor amino acids. Albert *et al.* (1949) made studies with a-estradiol labeled with radioactive iodine (I^{131}). This iodo-a-estradiol was found to be inactive physiologically, which casts doubt upon the validity of their findings. They observed localization of this material in the liver, salivary glands, mammary gland, skin, and fat of female mice, and possibly also in the uterus, adrenals, ovary, and pituitary. Of these tissues the mammary glands showed by far the largest uptake.

Lewison *et al.,* (1951) studied the tissue localization of estrone sulfate labeled with radioactive sulfur (S^{35}) in rats and found possible evidence of localization in mammary gland, liver, ovary, kidney, uterus, and vagina. This work is complicated and possibly invalidated by lack of information as to the amount of hydrolysis of the estrone sulfate which took place during the experimental period. It is of interest to note that these investigators found what they considered to be conclusive evidence of estrone sulfate localization in normal women in the epithelium of the genital tract and in the breast. It is remarkable to note that the highest localization found occurred in a breast cancer. On the other hand Davis *et al.* (1950) were unable to demonstrate localization of estrone sulfate labeled with radioactive sulfur (S^{35}).

This work on the tissue localization of hormones labeled with radioactive elements gives valuable information as to the target organ of the particular hormone. Such findings, presented in Fig. 1, illustrate the scope of this work. The applications of this technique to the investigation of the point of hormone action in the cell are also reviewed.

METHODS

Isotopic Labeling

The review of literature mentioned results obtained using two powerful new biochemical tools, isotopic tagging of biologically active materials and tissue homogenate fractionation procedures. We shall now examine their possible application to our present problem—that of determining the initial point of activity of the lactogenic hormone in the mammary gland secretory cell.

Radioactive isotopes were available to only a handful of workers 15 years ago when all such material was produced at great expense, when and if one of the half dozen cyclotrons in the country was available. With the beginning of the second world war all such investigation was halted, not to

be resumed again until 1946. Since that time, various radioactive isotopes have been available in quantity and at moderate cost from the government's atomic piles.

The report of Stadie *et al.* (1949a) on insulin binding in diaphragm muscle suggested the possibility that this phenomenon of tissue binding might be requisite for protein hormone action. Cox (1951) and Sonenberg *et al.* (1951), who worked with lactogenic hormone labeled with radioactive iodine (I^{131}), reported a tissue localization which gave further support to this idea and suggested that the best approach to the problem might lie in the use of lactogenic hormone labeled with radioactive iodine (I^{131}).

Various studies of the reaction of the protein lactogenic hormone with iodine have been made. Li *et al.* (1941) reported that in phosphate buffer of pH 7.0, iodine reacts only with the tyrosine groups in the lactogenic molecule to form diiodotyrosine groups. Their data suggest the presence of two types of tyrosine groups in the lactogenic molecule which react with iodine at different rates. This may be important in trace labeling techniques since one could then assume that all of the more reactive tyrosines would combine with iodine before many of the less reactive groups were affected. Li *et al.*, (1940a) also reported that upon extensive iodination of the lactogenic hormone all biological potency was lost. Thus, since we must retain biological activity in the hormone preparation, it becomes necessary that we trace-iodinate only a few of the 4.5-5.7 percent tyrosine groups present (Li *et al.*, 1940b). This would amount to iodinating no more than one or two of the approximately ten tyrosine groups present per molecule. This criteria is met by using the carrier-free radioactive iodine (I^{131}) solution as received from the Atomic Energy Commission in which the actual iodine content is negligible.

The procedure used here for the labeling of lactogenic hormone with radioactive iodine (I^{131}) was similar to that of Stadie, Haugaard, and Vaughan (1952) for the labeling of insulin. This procedure appeared to be highly efficient in giving them a product with a high specific activity, activities of 5-9 x 10^6 c.p.m. per mg. insulin compared to older work in which the synthesis yielded labeled insulin with an activity of 6 x 10^3 c.p.m. per mg. The following is the synthetic procedure which was followed.

Eight to ten millicuries of radioactive iodine (I^{131}) from Oak Ridge were transferred to a 25 x 400 test tube in which had been placed two drops of a solution of 0.07 M ferrous sulfate and 0.05 M ferric sulfate in 1.0 M sulfuric acid. This mixture was allowed to stand for five minutes. Five milligrams of lactogenic hormone dissolved in approximately five milliliters of 0.2 M phosphate buffer pH 7.4 were added to the radioactive iodine mixture. This mixture was allowed to stand for two hours at room temperature (approximately 25° C) with occasional air stirring. The reaction mixture was

transferred to a cellophane sac and dialyzed against running tap water for 72 hours and then 12 hours against fixed volume of isotonic sodium chloride. The labeled hormone preparation was then diluted to the necessary volume for injection.

Unless otherwise noted, the lactogenic hormone preparation used in this work was Squibb preparation #71713. This was stated to assay 20-25 I.U. per mg., of lactogenic activity, 0.02 I.U. of ACTH activity per mg., and less than one percent each of growth, FSH, and thyrotrophin activity.

Treatment and Injection Procedure

It was felt that rats would not be suitable for this type of work. The small amount of mammary gland tissue available from fully developed animals, even at the peak of lactation, would present problems of fractionation technique and from previous experience it had been found that homogenization of rat mammary tissue was exceedingly difficult. The rabbit was the next laboratory experimental animal in size that was available.

This animal has a mammary gland development of sufficient size to allow the contemplated experimental procedures. We were, however, faced with the problem of administration of the labeled hormone. With the rabbit, the minimal effective systemic dose is approximately 75 I.U., which is close to the amount of material we were able to synthesize at any one time. However, the rabbit has been used as an assay animal for the lactogenic hormone in another manner. This general procedure, which was proposed by Lyons (1942), was used with some variation. Lyons (1942) reported positive lactational response in all glands injected intraductally with three international units of lactogenic hormone. Meites and Turner (1948), using pseudopregnant rabbits in this manner, found ten international units gave a positive response in all glands injected.

Mature rabbits were injected with 50 to 75 I.U. of chorionic gonadotropin to induce pseudopregnancy. They were used experimentally 14 or 15 days later. The hair over the mammary region was clipped off while the animals were tied to an operating board, ventral side up. With the aid of dissecting glasses, the intraductal injections were made using a 1.0 milliliter tuberculin syringe and 27-gauge hypodermic needle. The needle had been cut down to about 1 centimeter in length and smoothed off without a bevel. The injections were 0.5 milliliters of lactogenic-I^{131} in isotonic saline calculated to be approximately 5 I.U. Generally four or five ducts in each of four or five teats were injected, for a total of approximately 20 injections.

Sixty to 75 minutes after the first injection, the experimental animal was sacrificed by means of an intravenous injection of 10 ml. of air. The mammary glands were then dissected free and placed in ice-cold isotonic sucrose for transfer to the cold room for subsequent handling.

Tissue Fractionation Procedures

DeDuve and Berthet (1954) published an excellent critical survey of the procedure of differential centrifugation and associated techniques, giving the successful applications and the limitations of the method. To quote a general statement from this survey:

"Differential centrifugation has been applied successfully to a number of different organs and, although some tissues are less suitable than others owing to their heterogenous cellular content or tough connective framework, the indications are that approximately the same techniques can be used with most types of biologic material."

These workers believe that as long as certain precautions are observed almost any tissue may be handled by these methods. The precautions are:

"To be suitable for centrifugal fractionation, the homogenate should (a) be free of extraneous elements such as blood and connective tissue; (b) contain all the particulate components of the tissue's cells in a state of morphologic and chemical integrity, as well as in one of perfect division from each other and from the soluable components."

Methods used in this investigation for tissue homogenization and fractionation will be considered in terms of those general precautions.

The mammary gland tissue as excised from the experimental animal was taken from the ice bath and ground in a meat grinder. This and all subsequent homogenization and fractionation treatment was carried out in the cold (4°-6° C). The grinding process accomplishes two purposes: (a) breakup of the resistant network of connective tissue in the gland to a great enough extent that homogenization is possible and (b) breakup of the duct system so that injected tagged lactogenic hormone which is not absorbed may be removed before homogenization.

The next step in this procedure involved removal of this unabsorbed, labeled material from the ground-up mammary tissue. This was accomplished by washing five times with 100 milliliter portions of isotonic sucrose. The choice of isotonic sucrose will be explained when the homogenization technique is discussed and the use of five washes will be justified on an experimental basis when results are given.

There are two major methods for the homogenization of tissues, both of which depend upon a mechanical rupture of the cells. These two methods are the Potter homogenizer as developed by Potter and Elvehjem (1936) which ruptures the cells by friction between a moving and a still surface, generally a glass tube and pestle; and the various devices such as the Waring Blendor which rupture the cells by mechanical shearing between rapidly rotating blades. Schneider and Potter (1949), Hogeboom (1951), Walker (1952), DeDuve *et al.* (1953) and others have all criticized the Waring blendor as causing excessive damage to the particulate components of the cells.

The Waring blendor had to be used in this investigation since previous experience had shown that the Potter homogenizer was unable to handle the tough connective tissue present in the mammary gland when large quantities of tissue (25-35 gm.) were used and give a high enough percent breakage of the epithelial cells of the gland.

An important consideration in such study as this is the choice of suspension medium. Much work has been done which indicates that isotonic sucrose media, as first introduced by Schneider, (1948) are probably the best for most purposes. This was the media chosen.

The washed mammary gland was first mixed with a volume of isotonic sucrose necessary to give a final 20 percent homogenate and then homogenized for two minutes at 75 percent speed and one minute at full speed in the Waring blendor. This gave reasonably good homogenization of the tissue without undue heating of the homogenate. Homogenization was followed by a filtration to remove as much as possible of the connective tissue and unbroken clumps of cells. The homogenate was filtered through first one, then two, and finally four layers of cheese cloth and this filtered homogenate was used for the subsequent fractionations.

DeDuve and Berthet (1954) note in their review that in general the fractionation procedures with isotonic sucrose homogenates have used forces of 600 g. for 10 minutes to 1000 g. for 10 minutes for the separation of a crude nuclear fraction; the large granule fraction, the mitochondria, has been isolated with forces of 5,000 g. for 10 minutes to 16,000 g. for 10 minutes; the microsomes have been isolated at forces of 1.0×10^6 to 1.8×10^6 g-min. (force in gravities times time applied). However, they recommend forces of the order of 3×10^6 g-min. for complete sedimentation.

It has been noted by several workers that the crude nuclear fraction obtained by these methods, Schneider and Hogeboom (1950), is grossly contaminated with cytoplasmic material, connective tissue, erythrocytes, and cells which escaped rupture (Novikoff *et al.*, 1953; Hogeboom *et al.*, 1952; Palade, 1951). From initial experiments it appeared that this situation would have to be corrected and thus methods were sought which would permit the nuclear fraction to be obtained in more pure form. The procedure for the separation of nuclei proposed by Wilbur and Anderson (1951), involving separation across layers of sucrose solution differing in density, appeared to be best suited to this problem and was thus adopted.

The fractionation used, except as noted, was as follows: Portions of approximately 20 milliliters of the filtered 20 percent homogenate were centrifuged for 10 minutes at 1000 g. to sediment the crude nuclear fraction. The supernatant (S la) was carefully decanted off and saved. The sediment was resuspended in isotonic sucrose and washed twice at 1000 g. for 10 minutes, the supernatants (S lb, S lc) being saved and all (S la, b, and c) combined and saved as (S l) for future fractionation.

The crude nuclear fraction was then resuspended in fifteen milliliters of 0.145 M sucrose and carefully layered over 25 milliliters of 0.218 M sucrose and centrifuged for eight minutes at 34 g. The upper 20 milliliters of supernatant were then removed by pipetting and layered over 25 milliliters of 0.272 M sucrose and centrifuged for eight minutes at 500 g. to sediment the nuclei. The upper 20 milliliters of supernatant (S 2a) were saved. The sedimented nuclei were resuspended in 15 milliliters of 0.145 M sucrose, layered over 25 milliliters of 0.272 M sucrose and centrifuged for eight minutes at 500 g. The upper 20 milliliters of supernatant (S 2b) were saved and combined with S 2a to give supernatant S 2; the remainder of the supernatant was carefully removed to leave the purified nuclear fraction.

Supernatants S 1 and S 2 were combined and centrifuged at 8,500 g. for 10 minutes to sediment the mitochondria. The supernatant (S 3a) was decanted off, the mitochondria resuspended in 10 milliliters of isotonic sucrose and washed twice as above at 8,500 g. for 10 minutes, supernatants S 3b and S 3c being saved and combined with S 3a to give supernatant S 3 and the mitochondrial fraction.

The supernatant S 3 was then centrifuged at 25,000 g. for two hous to sediment the submicroscopic particles, the microsomes. The supernatant S 4a was saved, the microsomes being resuspended in 25 milliliters of isotonic sucrose and recentrifuged at 25,000 g. for 90 minutes. This yielded the microsomal fraction and the supernatant S 4b was combined with S 4a to yield the final supernatant.

This procedure gave a purified nuclear fraction, a mitochondrial fraction, a microsomal fraction, and a final supernatant fraction.

Methods of Measurement

The various samples obtained from the fractionation procedure were digested in a minimum volume of 1 M KOH on a boiling water bath. HCl was added to the KOH digest to faint alkalinity (phenolphathalein). Duplicate aliquots of the digest were evaporated to dryness in counting cups under an infra-red heat lamp. The samples were then held for 24 hours in a dessicator to insure dryness.

The dried samples were counted with a scintillation detector (Anthracene crystal) connected to either a scale of 64 or a scale of 1000 scaler. All samples were counted for a least 2000 counts for a precision of at least 3 percent. All samples were corrected for self-absorption where it was not possible to hold the sample size constant. In phases of the work where this was possible, self-absorption corrections were not made.

Chemical Fractionation Procedures

Three type of chemical treatment have been used in this work and will be described in detail; several other procedures will be detailed where results

from use of them are given. Two of these methods deal with the isolation of nucleoprotein while the last is adaptable to the partial purification of nucleoprotein.

Volkin and Carter (1951) in a study of RNA used two M guanidine hydrochloride to precipitate ribonucleoprotein from a tissue extract. It was found that the RNA obtained was still highly polymerized and apparently only little changed. Grinnan and Mosher (1951) modified somewhat the above procedure and used it with liver for the production of a highly polymerized and apparently undenatured RNA. Their procedure is essentially one of homogenization, centrifugation to remove tissue debris and nuclei, and precipitation of the nucleoprotein with 4 M guanidine hydrochloride at pH 6.5. They found that the precipitated nucleoprotein was not dissociated during dialysis against water.

In the present study this procedure was slightly modified and used as follows: The mammary gland homogenate in isotonic sucrose was centrifuged at 1000 g. for 10 minutes to remove nuclei and cellular debris. This preparation, hereafter referred to as the "unfractionated particulate preparation", was then taken to 4 M concentration of guanidine hydrochloride and the pH rapidly adjusted to 6.5-7.0 with dilute NaOH. This solution was held at 38°-40° C for one hour, chilled rapidly, and held at 0-2° C for at least one hour. Following this the precipitate of nucleoprotein was removed by filtration or by centrifugation.

Various investigators (Cohen, 1946; Cohen, 1947; Berkman, Housewright, and Henry, 1949; Euler and Heller, 1948) have studied the precipitation of nucleic acids and nucleoproteins with streptomycin. Euler and Heller (1948) found that liver nucleoproteins (mitochondria and microsomes) were strongly precipitated at pH 7.1-7.4 by M/400-M/1600 streptomycin. In this work the homogenate, as prepared above free of nuclei, was brought to pH 7.0-7.4 if not already at this pH, and streptomycin was added to a final concentration of M/200 to M/400. The solution was held at room temperature for one hour with occasional shaking, chilled to 0° C for one hour and the nucleoprotein precipitate removed by centrifugation or filtration.

Sevag *et al.,* (1938) used chloroform to remove free protein from nucleic acid solutions, the protein and nucleic acid being maintained in what was apparently the native condition. To the solution of nucleic acid and free protein $^1/_4$ volume of chloroform was added and the mixture shaken for one hour. Usually $^1/_{10}$ volume of amyl alcohol was added to prevent foaming during this shaking process. The mixture was centrifuged, resulting in three layers, chloroform on the bottom then a layer of chlorofrom-protein three layers, chloroform on the bottom then a layer of chloroform-protein gel, and finally the top layer of nucleic acid in aqueous solution. This separation was found to be highly efficient at removing the protein from nucleic acid solutions.

In the course of the present investigation this procedure was varied rather extensively so that it might be utilized, if possible, for the separation of nucleoprotein from free protein in solution. Grinnan and Mosher (1951) had indicated that the splitting of their liver ribonucleoprotein was difficult with chloroform and that for very complete separation of the protein and nucleic acid, six hours of shaking at room temperature was necessary. The procedure used here follows: the guanidine hydrochloride precipitated "labeled lactogenic hormone-nucleoprotein complex" taken into solution to the greatest extent possible was the material subjected to this extraction. The extraction was carried out in the cold at 5°-10° C. The solution to be extracted was placed in a column and chloroform was allowed to drip through the solution at a rate of 2 milliliters per minute for 12 minutes. The chloroform and chloroform-protein gel was drawn off continuously at the bottom. After removal of the extracted solution any chloroform-protein gel which was clinging to the column was also removed and added to that drawn off. By this process free protein was removed from solution, presumably without extensive rupture of the nucleoprotein.

Methods used for single experiments are described in the presentation of experimental results that follows.

RESULTS

Determination of Self-Absorption of Samples

The first information obtained in this study was a determination of the correction factors for self-absorption in the samples to be run. Since the sample weight could not, in all cases, be held constant, it was necessary to determine the amount of self-absorption which would occur under the conditions of sample preparation to be used, for the range of sample weights that would be encountered.

A rabbit mammary gland homogenate was made up and a known amount of radioactive iodine (I^{131}) added. Varying amounts of this homogenate were carried through the standard digestion, drying, and counting procedure and the counts per minute (c.p.m.) as experimentally determined by this procedure were compared to the c.p.m. found for the same amounts of radioactive iodine (I^{131}) standards of negligible weight. The self-absorption curve thus obtained is shown in Figure 2. It will be noted that infinite thickness for counting purposes is reached at a sample weight of 0.6 grams (122.2 milligrams per square centimeter). In this investigation all studies which have necessitated variable sample weights have been corrected for self-absorption from this curve. In portions of this investigation all sample weights were held essentially constant and were greater than 0.6 grams; therefore, these samples have not been corrected for sample self-absorption.

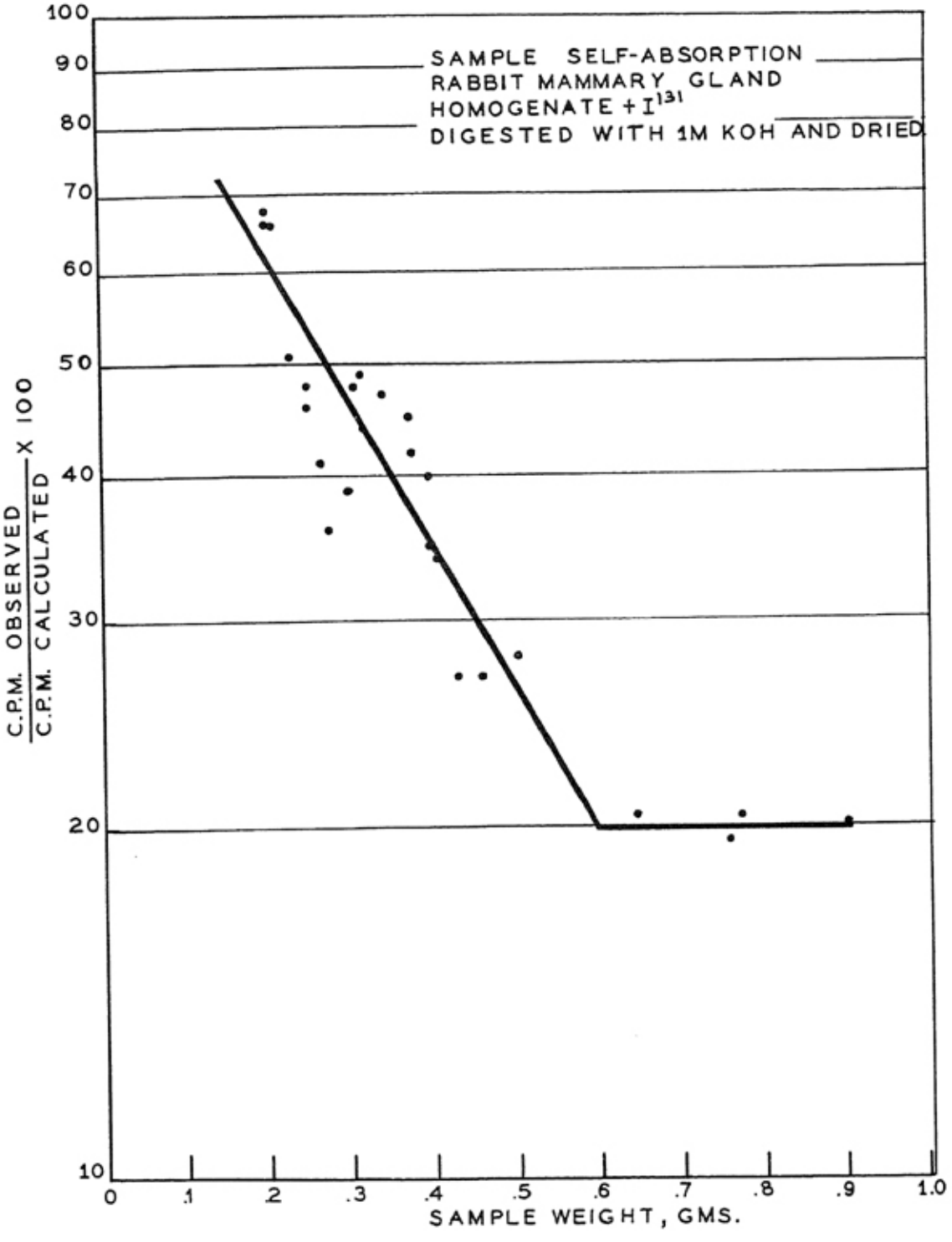
SAMPLE SELF-ABSORPTION
RABBIT MAMMARY GLAND
HOMOGENATE + I^{131}
DIGESTED WITH 1M KOH AND DRIED
C.P.M. OBSERVED / C.P.M. CALCULATED X 100
100
90
80
70
60
50
40
30
20
10
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
SAMPLE WEIGHT, GMS.

Figure 2

Period of Maximum Uptake and Number of Washes Necessary to Remove Unabsorbed, Labeled Hormone

An estimate of two conditions was necessary to the successful application of this method of intraductal injection of the tagged lactogenic hormone to the problem of cellular localization of the hormone.

First it was necessary to obtain an estimate of the speed with which the intraductally injected hormone was taken up by the mammary gland tissue. It was found (Table 1) that this uptake was fairly rapid. Four ducts in each

TABLE 1 -- UPTAKE AS RELATED TO LENGTH OF TIME POST-INJECTION

Time post-injection	Counts/minute/gm. tissue
30 minutes	12,989
1 hour	6,399
3 hours	1,985*
6 hours	2,430

*poor injection

of four glands were injected with equal volumes of lactogenic hormone labeled with radioactive iodine. The injections into each gland were staggered so that when the animal was sacrificed one gland had been injected for six hours, another for three hours, another for one hour, and one for 30 minutes. The glands were dissected free separately, ground up, weighed, washed at least five times with isotonic sucrose, digested, dried, and counted. The results from this experiment indicate a rapid uptake of the lactogenic hormone from the duct system which reaches a maximum at less than one hour and possibly less than 30 minutes following injection into the duct system.

The second estimate necessary to facilitate future experiments was the efficiency of the washing process with isotonic sucrose to remove unabsorbed, tagged lactogenic hormone remaining in the duct system of the mammary gland at the time that the experimental animal was sacrificed. This was estimated in the same experiment in which an estimate of the uptake time was obtained. Each gland when removed from the rabbit was divided in half after grinding and weighed. The ground-up mammary tissue was then washed with three, five, or eight changes of 100 milliliters of isotonic sucrose and prepared for counting. The results of this experiment show that washing with five 100 milliliter changes of isotonic sucrose appears to remove the majority of unabsorbed material from the ground-up glandular tissue (Table 2).

TABLE 2 -- ACTIVITY AS RELATED TO NUMBER OF WASHES IN ISOTONIC SUCROSE

Counts/minute/gram tissue		
Three changes Isotonic sucrose	Five Changes Isotonic sucrose	Eight Changes Isotonic sucrose
29,383	12,989	
7,628		6,399
	1,985	1,679

From these two estimates it was decided that for future experiments the experimental animals would be sacrificed approximately one hour after the first injection into the duct system. Due to the number of injections into the duct system made in each experiment and to variation in the case of injection, the length of time to which a particular gland segment was subjected to the influence of the labeled lactogenic hormone varied from approximately 30 minutes to somewhat more than one hour in a few experiments. The number of washes of the ground-up mammary gland tissue was held constant at five in all experiments.

Intracellular Localization of Labeled Lactogenic-I^{131} Hormone in Cytological Fractions of the Rabbit Mammary Gland

Localization of high purity lactogenic-I^{131} hormone: The initial study of the cytological localization of the I^{131} labeled lactogenic hormone was made using the methods previously described, the fractionation technique being essentially that of Schneider and Hogeboom (1950). This study (Table 3) indicates that localization of the I^{131} labeled lactogenic hormone

TABLE 3 -- CYTOLOGICAL LOCALIZATION OF ACTIVITY IN RABBIT MAMMARY GLAND FRACTIONATED BY THE METHOD OF SCHNEIDER AND HOGEBOOM (1950)

Cytological Fraction	Counts/minute	Percent Total activity
"Crude" nuclear	108,847	36.3
Mitochondrial	137,705	45.9
Microsomal	39,863	13.3
Supernatant	13,191	4.4
Totals	299,606	99.9

does occur in the rabbit mammary gland cell. From literature previously cited and from microscopic examination of the fractions obtained by this fractionation procedure it appeared that the nuclear fraction was grossly contaminated with unbroken cells, and with clumped mitochondria, and that this contamination might account for the high activity associated with these "crude" nuclear fractions.

On the basis of these results, the full fractionation procedure was adopted. The results of six experiments using these techniques are presented in Table 4. These results indicate that a major portion of the I^{131} tagged lactogenic hormone is associated with the particulate portions of the mammary gland cell, the mitochondria and microsomes, at an average time after injection of approximately 50 minutes. Only small amounts of activity are found with the nuclear fraction and amounts of this order may be due to particulate contamination. The activity noted in the supernatant fraction is small compared to the total activity and may be the result of several factors: labeled lactogenic hormone in the cytoplasm but not associated with cytoplasmic

TABLE 4 -- CYTOLOGICAL DISTRIBUTION OF I^{131}-LABELED LACTOGENIC HORMONE IN RABBIT MAMMARY GLAND ONE HOUR AFTER INTRADUCTAL INJECTION

Cytological Fractions	Experiment Number 1		2		3		4		5		6		Avg. per cent
	c.p.m.	% total	c.p.m.	% total	c.p.m	% total	c.p.m	% total	c.p.m.	% total	c.p.m.	% total	
Nuclear	2,034	5.2	2,814	6.9	796	3.6	936	4.7	5,062	6.6	1,456	5.4	5.4±1.3
Mitochondrial	22,833	58.1	26,622	65.1	9,142	41.8	8,695	43.7	41,480	54.7	13,430	49.8	52.2±9.3
Microsomal	12,298	31.3	9,196	22.5	9,849	45.1	8,022	40.3	18,162	23.9	6,207	23.0	31.0±9.0
Supernatant	2,160	5.5	2,273	5.6	2,063	9.4	2,254	11.3	11,111	14.6	5,835	21.6	11.3±6.4
Total Particulate	35,131	89.4	35,818	87.6	18,991	86.9	16,717	84.0	59,642	78.6	19,637	72.8	83.2±6.6
Totals	39,325	100.1	40,905	100.1	21,850	99.9	19,907	100.0	75,815	99.8	26,928	99.8	99.9

structures, unabsorbed labeled lactogenic hormone which was not removed by the washing procedure, labeled lactogenic hormone which was actually associated with particulates of the cytoplasm but which was not sedimented by the fractionation procedure, and, finally, split products of the labeled hormone carrying the I^{131} or free I^{131} itself. In connection with this latter possibility it might be mentioned that the spleen, liver, and thyroid of the experimental animals showed only very low levels of activity when examined after sacrifice of the animal.

Localization of lactogenic-I^{131} hormone at a shorter time interval after injection: One set of experiments was carried out in which, the animal was sacrificed 45 minutes after the initial injection was made. This reduced the time in which the hormone preparation was in contact with the mammary gland to 45 minutes at the most and 10 to 15 minutes at the least. This would give an average of approximately 28 minutes for uptake into the mammary gland cell, compared to the previous experiments in which the labeled lactogenic hormone was in the duct system for an average of approximately 50 minutes. These results (Table 5) seem to indicate a difference in

TABLE 5 -- CYTOLOGICAL DISTRIBUTION OF ACTIVITY IN MAMMARY GLANDS OF RABBITS SACRIFICED AT SHORTER TIME INTERVALS (45 MIN. POST-INJECTION)

Cytological Fractions	Experiment Number 1		Experiment Number 2		Average Percent
	c.p.m.	% total	c.p.m.	% total	
Nuclear	16,297	4.8	32,062	11.3	8.1
Mitochondrial	129,470	38.8	67,739	24.0	31.4
Microsomal	39,310	11.8	49,304	17.5	14.7
Supernatant	147,999	44.4	132,333	47.0	45.7
Total Particulate	168,780	50.6	117,043	41.5	46.1
Totals	333,076	99.8	281,438	99.8	99.9

the pattern of distribution with time. This is not surprising; it seems to substantiate the idea that the phenomenon being studied here is a dynamic one related to the metabolism and function of the cell. While these limited data can only be suggestive it would appear that the labeled lactogenic hormone may first enter the supernatant portion of the cell and then become associated with the particulates. In other words, the free hormone appeared first to be transported across the cell wall into the intracellular fluid and then to become associated with the cytoplasmic mitochondria and microsomes. These data also seem to give indication that the association of the tagged hormone takes place more rapidly with the mitochondria than with the microsomes.

Localization of lower purity lactogenic-I^{131} hormone: Two experiments were conducted using a different lactogenic hormone preparation which was of lower purity than that used in the bulk of this work. This

preparation was stated as assaying 15-20 international units of lactogenic hormone per milligram, 0.084 U.S.P. units of oxytocin per milligram, 0.5 units of pressor activity per milligram, 6.2 ± 1.9 percent of growth hormone, 0.1 c.u. of gonadotrophin per milligram, and 0.046 ± 0.013 units of thyrotrophin per milligram. A vastly different pattern (Table 6) of distribu-

TABLE 6 -- CYTOLOGICAL DISTRIBUTION OF A SECOND LACTOGENIC HORMONE PREPARATION IN THE RABBIT MAMMARY GLAND

Cytolegical Fractions	Experiment Number 1		2		Average Percent
	c.p.m.	% total	c.p.m.	% total	
Nuclear	7,266	31.2	8,166	30.8	31.0
Mitochondrial	7,344	31.5	10,438	39.4	35.5
Microsomal	4,410	18.9	4,416	16.6	17.8
Supernatant	4,265	18.3	3,450	13.0	15.7
Total Particulate	11,754	50.4	14,854	56.0	53.2
Totals	23,285	99.9	26,470	99.8	100.0

tion in the mammary gland fractions at one hour is found with this lactogenic hormone preparation. It is assumed that this difference is due to the lesser degree of purity of the preparation, compared to the lactogenic hormone which was used in the bulk of the work reported. If this is true, these results are due to the higher percentage of miscellaneous proteins present, which became labeled in the synthesis with radioactive iodine, obscuring the pattern of lactogenic hormone distribution. While this may not be the case, results presented below make it the most likely explanation.

Intracellular Distribution of Radioactive Iodine (I^{131}) in Cytological Fractions of Rabbit Mammary Gland

The results which have been presented for studies with the labeled lactogenic hormone preparations might conceivably have been due to the presence of free radioactive iodine. This possibility could have come about in several ways: incomplete removal of free radioactive iodine during the dialysis following synthesis of the tagged lactogenic hormone, splitting of the tagged lactogenic hormone in the duct system to release free radioactive iodine, or splitting of the tagged lactogenic hormone after it has entered the mammary gland cell to release free radioactive iodine. Several experiments were performed to determine whether this had occurred to any great extent and could have been responsible for the results.

Dilute solutions of radioactive iodine in isotonic saline were injected intraductally into the rabbit mammary glands in the same manner used with the labeled lactogenic hormone. The purpose was to determine the pattern of distribution which would be obtained with the free radioactive iodine. In contrast to the results with labeled lactogenic hormone, the great majority of activity was found in the supernatant fraction following injection of the free radioactive iodine (Table 7). These results seem to eliminate the pos-

TABLE 7 -- CYTOLOGICAL DISTRIBUTION OF I^{131} INJECTED INTRADUCTALLY INTO RABBIT MAMMARY GLAND

Cytological Fractions	Experiment Number 1		2		Average Percent
	c.p.m.	% total	c.p.m.	% total	
Nuclear	596.0	7.5	638.6	8.2	7.9
Mitochondrial	162.2	2.0	211.6	2.7	2.4
Microsomal	209.6	2.6	113.4	1.4	2.0
Supernatant	6,966.0	87.8	6,824.0	87.6	87.7
Totals	7,933.8	99.9	7,787.6	99.9	100.0

sibility that the results obtained with the labeled lactogenic hormone are actually due to free radioactive iodine distribution in the mammary gland cell. They do not rule out the possibility that the activity found in the supernatant fraction is due to free radioactive iodine.

Following the intraductal injection of free radioactive iodine, the liver, spleen and particularly the thyroid showed high activity, indicating that radioactive iodine was passing into the general circulation of the animal. This was not the case following injection of the labeled lactogenic hormone.

Intracellular Localization of Radioactive Iodine Labeled Homologous Serum Proteins

Several experiments were conducted in which rabbit serum proteins were labeled with radioactive iodine and injected intraductally, as previously described, and the cytological distribution determined. This material was obtained from the synthesis as given above, using salt precipitated rabbit serum proteins with no further purification. The experimental animals were sacrificed one hour after the initial injection.

The data (Table 8) indicate some differences in the cytological distribution of this material, compared to the labeled lactogenic hormone. The serum proteins show a higher concentration of activity in the nuclear and supernatant fractions and a lower concentration in the microsomal and total particulate fractions. Apparently a somewhat different pattern of distribution occurs in the mammary gland cell with homologous serum proteins. This may suggest that the pattern of distribution which is found with the labeled lactogenic hormone is specific for that hormone.

TABLE 8 -- CYTOLOGICAL DISTRIBUTIONS OF I^{131}-LABELED RABBIT SERUM PROTEINS INJECTED INTRADUCTALLY INTO RABBIT MAMMARY GLAND

Cytological Fractions	Experiment Number 1		2		Average Percent
	c.p.m.	% total	c.p.m.	% total	
Nuclear	5,945	6.6	9,253	13.5	10.1
Mitochondrial	54,731	61.5	34,600	50.6	56.1
Microsomal	15,390	17.3	10,637	15.5	16.4
Supernatant	12,826	14.4	13,856	20.2	17.3
Total Particulate	70,121	78.8	45,237	66.1	72.5
Totals	88,892	99.8	68,346	99.8	99.9

Exchange of Localized Labeled Lactogenic Hormone With Added Unlabeled Lactogenic Hormone

It was thought that the amount of shift of activity from one fraction to another, which might occur upon the addition of a large excess of lactogenic hormone to the mammary gland homogenate before fractionation, might indicate something of the nature of the distribution which occurs *in vivo* following the intraductal injection of the labeled lactogenic hormone. It might be possible to determine in this manner if the distribution was due to adsorption, since, if this were the case, the addition of a large excess of hormone (10 milligrams) would be expected to displace a large percentage of the labeled lactogenic hormone so that it would appear in the supernatant fraction.

While the results (Table 9) show that some increase in the supernatant activity occurs following incubation of the mammary gland homogenate with excess unlabeled lactogenic hormone, this increase and the corresponding decrease in activity associated with the particulates apparently are not large enough to represent a great degree of particulate association due to adsorptive phenomenon.

Distribution of Activity as Associated with Cytoplasmic Nucleoprotein

The studies reported above have indicated a marked association of the radioiodine labeled lactogenic hormone with the particulates of the cytoplasm of the rabbit mammary gland. It was deemed of interest to follow up these cytological studies with an attempt to determine something of the nature of the cytological association. First, studies were undertaken to obtain an indication of whether or not the radioactive iodine remained firmly attached to large protein molecules in its association with the cellular particulates.

These studies were carried out on the "unfractionated particulate preparation" (the supernatant containing the mitochondria, microsomes and nonparticulate material remaining after the removal of nuclei and debris from the isotonic sucrose homogenate by centrifugation at 1000 x g. for 10 minutes).

The "unfractionated particulate preparation" was treated with trichloracetic acid to 5 percent concentration in three experiments (Table 10). Results indicate that essentially all of the activity of these portions of the cell resides in protein molecules of a size large enough to be precipitated with 5 percent trichloracetic acid. Obviously then, this portion of the mammary gland homogenate contains very little free iodine or other metabolic breakdown product of the tagged lactogenic hormone.

TABLE 9 -- CYTOLOGICAL DISTRIBUTION OF ACTIVITY IN HOMOGENATES BEFORE AND AFTER ADDITION OF UNTAGGED LACTOGENIC HORMONE

Cytological Fractions	Experiment No. 1					Experiment No. 2				
	Before Addition		After Addition		Percent	Before Addition		After Addition		Percent
	c.p.m.	% total	c.p.m.	% total	Change	c.p.m.	% total	c.p.m.	% total	Change
Nuclear	5,062	6.6	3,920	5.1	-22.7	1,456	5.4	2,492	11.2	+107.4
Mitochondrial	41,480	54.7	31,517	41.1	-24.9	13,430	49.8	7,230	32.5	- 34.7
Microsomal	18,162	23.9	22,301	29.1	+21.8	6,207	23.0	4,953	22.2	- 3.5
Supernatant	11,111	13.6	18,845	24.6	+68.9	5,835	21.6	7,571	33.9	+ 56.9
Total Particulate	59,642	78.6	53,818	70.2	-10.7	19,637	72.8	12,183	54.7	- 24.9
Totals	75,815	99.8	76,583	99.9		26,928	99.8	22,246	99.8	

TABLE 10 -- PRECIPITATION OF ACTIVITY FROM UNFRACTIONATED PARTICULATE PREPARATIONS

Experiment								
4M Guanidine HCL ppt'ion			Streptomycin ppt'tion			5% TCA ppt'ion		
ppt. cpm	s'nat.cpm	% ppt.	ppt. cpm	s'nat.cpm	%ppt.	ppt.cpm	s'nat.cpm	% ppt.
243,867	120,070	67.0	476,015	113,406	80.7	61,024	2,999	95.3
71,531	53,388	57.2	206,666	35,995	85.1	32,266	528	98.4
76,482	29,284	72.3	111,549	14,886	88.2	23,082	340	98.6
206,240	36,873	84.8	82,860	10,702	88.5			
601,242	115,590	83.8						
375,056	137,538	73.1						
1,850	1,556	54.3						
27,806	4,988	84.7						
152,759	11,921	92.7						
Average ppt.	74.4±14.2			85.6±10.3				97.4

TABLE 11 -- SOLUBILIZATION OF GUANIDINE HYDROCHLORIDE PRECIPITATED "LABELED LACTOGENIC HORMONE - NUCLEOPROTEIN COMPLEX" WITH VARIOUS REAGENTS AS MEASURED BY NON-DIALYZABLE I^{131}

	Isotonic Sucrose				50% Ethanol	0.0034 N NH_4OH	.14 M NaCl
	#1	#2	#3	#4	#1	#1	#1
c.p.m. Soluble	12,000	3,597	7,193	4,818	3,347	21,128	4,379
c.p.m. Insoluble	113,350	107,952	208,730	22,204	29,118	180,330	148,380
Percent Soluble	9.6	3.2	3.3	17.8	10.3	10.5	2.9
Percent Insoluble	90.5	96.8	96.7	82.2	89.7	89.5	97.1

Further studies of the nature of this association of activity in the "unfractionated particulate preparations" were carried out using two different techniques for the precipitation of the nucleoprotein present. These methods were precipitation with four molar guanidine hydrochloride and with molar /200 to molar/400 streptomycin as previously outlined.

Data (Table 10) indicate strikingly that the cytological localization of the lactogenic hormone tagged with radioactive iodine in the particulate portions of the cell, the mitochondria, and the microsomes is, in fact, an association with the nucleoprotein of the cytoplasm. In the light of the chemical composition which has been found for mitochondria and microsomes from many sources, this finding is not surprising and, indeed, the data seem to complement one another to a great extent.

It should be noted that with these procedures little, if any, free labeled lactogenic hormone was precipitable. With the free labeled lactogenic hormone it was impossible to obtain a precipitate using the streptomycin procedure. Using the guanidine hydrochloride procedure, some activity was found in the precipitate but this was removed by the washing process with 4-molar guanidine hydrochloride solution and thus was not actually precipitated material.

Although only small amounts of the guanidine hydrochloride and streptomycin precipitated material were available, some qualitative chemical studies of acid hydrolyzates of this material were made. Positive biuret and xanthoproteic tests were obtained. Slightly positive tests were obtained for the presence of purine bases by precipitation as silver salts. Determination with orcinol gave strongly positive tests for pentose while tests for deoxypentose with diphenylamine were negative or only slightly positive. It was found in the course of one experiment that a hot 10 percent trichloracetic acid extract of the guanidine hydrochloride precipitated material contained only 3.4 percent of the total activity. This indicates that the radioiodine activity continued to reside in the protein portion of the precipitated nucleoprotein.

Attempts to Solubilize the Tagged Lactogenic Hormone-Nucleoprotein "Complex"

It was hoped that studies aimed at clarifying the nature of this association of the labeled lactogenic hormone and the cytoplasmic nucleoprotein, hereafter referred to as the "tagged lactogenic hormone-nucleoprotein complex," could be carried out. Two problems immediately beset such study; only small amounts of the "complex" were available at any one time and, as indicated by the amount of radioactivity in solution, this complex was highly insoluble.

To this end, studies of the solubility of the precipitated "tagged lacto-

genic-nucleoprotein complex" were initiated. A variety of solvents and procedures were utilized and will be reported.

Solubilization of the 4-molar guanidine hydrochloride precipitated "complex" was attempted with four solvents; 0.14 molar sodium chloride, 50 percent ethanol, 0.0034 normal ammonium hydroxide, and isotonic sucrose. In all cases approximately 100 milliliters of the solvent were used and the undissolved "labeled lactogenic hormone-nucleoprotein complex" removed by centrifugation at 10,000 x g. for 10 minutes. The supernatant was then dialyzed overnight against the solvent. None of these solvents took very large amounts of the precipitated "complex" into solution as determined by the radioactivity of the supernatant after centrifugation and dialysis (Table 11).

Following these relatively futile attempts, other methods that had been reported effective in various situations were evaluated for their usefulness as solvents for this material.

The first of these methods was suggested by the reports of Wainio and his associates (1948, 1953) dealing with the purification of cytochrome oxidase. They found that 2 to 4 percent sodium desoxycholate solutions had a marked effect upon solubilizing the cytochrome oxidase associated with the insoluble particulates of heart muscle preparations.

A second method which was attempted was one suggested by the report of Morton (1950) dealing with the solubilization of enzymes associated with cytoplasmic particulates. He reported that some of the enzymes associated with the insoluble cytoplasmic particulates would be brought into solution by treatment with aqueous solutions containing excess n-butyl alcohol.

A third method, which has been used in the past, involves subjecting the insoluble material to the action of strong sodium or potassium hydroxide at room temperature or in the cold. This treatment was tried, the cytoplasmic particulates being subjected to the action of 1 molar potassium hydroxide for 12 hours at room temperature.

In all experiments the treatment outlined above was followed. The "unfractionated particulate preparation" was treated with desoxycholate or n-butyl alcohol for three hours or with one molar potassium hydroxide for 12 hours, all at room temperature. Following this the material was centrifuged at 25,000 x g. for 90 minutes to remove any fine suspended material. The supernatant was then dialyzed overnight against distilled water and the streptomycin-precipitable material counted as being the material which was initially taken into solution. The results, using these methods (Table 12), are no more encouraging than the results reported for the solubilization of the "tagged lactogenic hormone-nucleoprotein complex".

The results suggest several possibilities. It is obvious that none of the methods resulted in solubilization as measured by activity precipitable with

TABLE 12 -- SOLUBILIZATION OF PARTICULATES AS MEASURED BY I^{131} ACTIVITY OF NON-DIALYZABLE, STREPTOMYCIN PRECIPITABLE MATERIAL

	4 Percent Desoxycholate	1 M KOH 12 hrs., 25°C		Excess N-Butyl Alcohol	
	#1	#1	#2	#1	#2
c.p.m. Soluble	17,760	6,765	9,357	9,450	7,953
c.p.m. unfractionated particulate preparation	423,620	423,620	190,530	423,620	292,035
Percent Soluble	4.2	1.6	4.9	2.2	2.7
Percent insoluble or dialyzed	95.8	98.4	95.1	97.8	97.3

streptomycin following dialysis. It is possible that some degree of solubilizatoin occurred with loss of the radioiodine label. That this might occur was indicated in one experiment using 1 molar potassium hydroxide in which 22.2 percent of the initial activity was present in the supernatant before dialysis and 4.9 percent was present in streptomycin precipitable form after dialysis.

The results reported here for n-butanol solubilization suggest that lipids are not involved in the association of the labeled lactogenic hormone with the cellular particulates (Morton, 1950; Reid, 1955). Reid (1955) has found that approximately 45 percent of the dry weight of liver cell particulates remains insoluble following n-butanol treatment, which suggests that the labeled lactogenic hormone association with the mammary gland cytoplasmic particulates is extremely stable. This study also suggested that denaturation of proteins occurs when the n-butanol procedure is carried out in aqueous solutions. This may also explain the low solubility noted.

Palade and Siekevitz (1955) have reported studies on rat liver microsomes which indicate that treatment with desoxycholate leaves an insoluble residue of approximately 22 percent of the original nitrogen, 79 percent of the original RNA, and only 8 percent of the original phospholipid. When these results are compared to the findings of the present investigation it again appears that the association of the labeled lactogenic hormone to the cytoplasmic particulates is highly stable and is not dependent upon the particulate lipids.

Effect of Chloroform Treatment on the "Labeled Lactogenic Hormone-Nucleoprotein Complex"

Several experiments were conducted using the chloroform treatment of Sevag (1938) as modified and described above. In these experiments the material used was the guanidine hydrochloride precipitated nucleoprotein in three of the solvents described: 0.14 molar sodium chloride, 50 percent ethly alcohol, and isotonic sucrose. The data (Table 13) from these experiments indicate that several possible actions may occur during this procedure. The treatment may have removed free labeled lactogenic hormone from the solu-

TABLE 13 -- EFFECT OF CHLOROFORM TREATMENT ON "LABELED LACTOGENIC HORMONE-NUCLEOPROTEIN COMPLEX" IN SOLUTION

	Experiment Number					
	1		2		3	
	0.14 M NaCl Extract		50% EtOH Extract		Sucrose Extract	
Fraction	c.p.m.	percent	c.p.m.	percent	c.p.m.	percent
Activity Removed by $CHCl_3$	68	16.5	488	4.3	3,945	37.8
Soluble Activity	343	83.5	10,785*	95.7	6,480**	62.2
Totals	411	100.0	11,273	100.0	10,425	100.0

*31.0 percent non-dialyzable, 12 hours
**74.4 percent non-dialyzable, 12 hours

tion and left predominately the hormone-nucleoprotein "complex" in the aqueous phase. It seems most likely that this process occurred. It is also possible that labeled lactogenic hormone of the "complex" was released by this procedure and finally it is probable that the protein denaturation which occurs at the chloroform-water interface resulted in the release of free radioactive iodine into the solution. It was found that six hours of treatment with chloroform at room temperature resulted in removal of 95 percent of the activity from the aqueous phase, which suggests that this treatment resulted in complete rupture of the "tagged lactogenic hormone-nucleoprotein complex."

Release of Dialyzable Radioactive Iodine from the "Tagged Lactogenic Hormone-Nucleoprotein Complex" and from Free Tagged Lactogenic Hormone

Though efforts to solubilize the "tagged lactogenic hormone-nucleoprotein complex" were almost fruitless, it was felt that some information as to the nature of this "complex" might be gained by studying the small amount of material which could be taken into solution. A comparison of the losses of radioactivity from solutions of the "complex" and from solutions of labeled lactogenic hormone in various solvents should give some indication of the comparative strength with which the radioactive iodine label is bound in the molecule. This comparison should give indication, then, as to whether the labeled lactogenic hormone has actually been bound to the nucleoprotein in the "complex", since if this be the case one might expect the relative bonding energies in the molecule to have changed. If differences are found, one other possibility is evident; that the radioactive iodine label may have changed position and may no longer be located on the lactogenic hormone molecule. This possibility cannot be ruled out, and indeed plagues all tracer studies where the end product is not or cannot be identified. This possibility will be discussed more fully in a later section.

These studies were carried out in several different solvent systems for three hours at room temperature. The solvent against which the material was being dialyzed was continually replaced. In practically all experiments the loss of radioactive iodine from the "labeled lactogenic hormone-nucleoprotein complex" upon dialysis amounted to 85 percent or more (Table 14).

TABLE 14 -- LOSS OF ACTIVITY DURING THREE HOUR DIALYSIS IN VARIOUS SOLUTIONS AT ROOM TEMPERATURE

Dialysis Solution	Lactogenic-Nucleoprotein "Complex" c.p.m. before	c.p.m. after	% loss	Free Lactogenic-I^{131} c.p.m. before	c.p.m. after	% loss
50% Ethyl Alcohol	10,785	3,347	69.0	25,879	20,445	21.0
50% Ethyl Alcohol	1,200	189	84.2			
Isotonic Sucrose	6,480	4,818	25.6			
pH 4 (HCL)	683	52	92.4			
pH 4 (HCL)	438	41	90.6			
pH 10 (NaOH)	683	81	88.1	25,879	20,625	20.3
pH 10 (NaOH)	438	91	79.2	641,635	509,705	20.6
pH 10 (NaOH)	1,200	174	85.5			
pH 10 (NaOH)	41	14	67.0			
Distilled Water	1,200	195	83.7	25,879	27,485	0
Distilled Water	720	113	84.3	31,476	31,140	1.1
Distilled Water	3,339	323	90.3			
Distilled Water	27,620	1,592	94.2			
0.1 M NaCl	683	60	91.2			
0.1 M NaCl	438	47	89.3			
0.5 M NaCl	41	23	45.3	641.635	304,633	52.6
0.5 M NaCl	1,200	120	90.0	25,879	15,150	41.5
0.5 M NaCl	3,339	308	90.8	31,476	21,340	32.2
0.5 M NaCl	27,620	1,830	93.4			
0.1 M NaCl	683	15	97.8			
0.1 M NaCl	438	20	95.4			

This high loss is quite striking, coming as it does from a material which has retained the activity through a precipitation procedure specific for nucleoproteins. In contrast, the loss of radioactive iodine from the tagged lactogenic hormone, treated in the same manner, amounts at most to 50 percent and in most experiments to much less. The actual loss from the tagged lactogenic hormone may be still less due to adsorption on the dialysis tubing as will be shown below. It would appear from these results then, that the radioactive iodine label is much less firmly bound in the "labeled lactogenic hormone-nucleoprotein complex" than in the labeled lactogenic hormone. This may be taken as an indication that bonding of some sort exists between the hormone and the nucleoprotein if the I^{131} label has remained attached solely to the lactogenic hormone.

Adsorption of Labeled Lactogenic Hormone on Cellophane Dialysis Tubing

The results of several experiments (Table 15) indicate that most, if not all, of the losses of radioactive iodine which occur upon dialysis of the labeled lactogenic hormone are due to adsorption. For these experiments, tagged lactogenic hormone was added to 0.14 molar sodium chloride and the activity of the solution determined before and three hours after the addition of the dialysis tubing. The data from these experiments indicates that approxi-

TABLE 15 -- ABSORPTION OF LABELED LACTOGENIC HORMONE BY DIALYSIS TUBING

	Experiment Number	
	1	2
c.p.m./ml. initial	1,664	2,015
c.p.m./ml. three hours	1,331	1,636
c.p.m./ml. decrease	333	379
Percent decrease	20.0	18.8

mately 20 percent of the activity was adsorbed. From Table 14 it would appear that this phenomenon does not occur in distilled water and that it may occur to an even greater extent in stronger solutions of electrolytes.

These data indicate that the comparative loss of radioactivity from the "complex" upon dialysis may have been greater than that previously noted. This tends to make the results (Table 14) even more striking.

DISCUSSION

Evaluation of Results

This investigation yielded two important sets of data which bear on the location of the point of action of the lactogenic hormone in the mammary gland secretory cell.

The cytological distribution of the intraductally injected, labeled lactogenic hormone is given in Tables 3, 4, and 5. Supplementary data relating to the distribution of radioactive iodine and tagged serum proteins is presented in Tables 7 and 8. From these data it appears that at least 80 percent of the labeled lactogenic hormone in the cell at the end of an average period of approximately 50 minutes post-injection is associated with the particulates of the cell. Smaller amounts of activity were found to be associated with the nuclear fraction and the supernatant fraction. Possible explanations for this were discussed under Results. The results reported in Table 5 also indicate particulate localization and suggest, although this point was not studied, that the time course of this phenomenon of cellular uptake and particulate association consists of entrance of the free hormone into the cell's cytoplasmic fluid, followed by association of the lactogenic hormone with the cell particulates.

The data for the cytoplasmic distribution of radioiodine in the mammary gland definitely indicates that the previous measurements have not been confused by the presence of large amounts of free I^{131}. The studies made with I^{131} labeled homologous serum proteins did not show a strikingly different distribution pattern in the mamary gland homogenates from that noted for the labeled lactogenic hormone. It does appear, however, that the activity of the nuclear fraction was higher and that of the microsomal and total particulate fractions somewhat lower. These small differences may indicate that a different pattern of cytological distribution exists for the

labeled lactogenic hormone, compared to the labeled serum proteins. If, as may be the case, these differences are more apparent than real it would suggest that a variety of homologous proteins may show the same intracellular distribution.

It is of interest also to compare the results of the serum protein distribution experiments (Table 8) with the results obtained with a lactogenic hormone preparation of lesser purity (Table 6). Although there are some marked differences in the distribution pattern, it may be seen that in relation to findings with a more pure lactogenic hormone that they tend to be similar. In both cases the nuclear and supernatant fractions are higher and the microsomal and total particulate fractions are lower. This comparison lends further support to the idea that the differences in distribution which were noted for the two lactogenic hormone preparations are due to differences in purity.

The set of experiments which were performed in an effort to determine the amount of exchange which might take place in mammary gland homogenates from animals injected with labeled lactogenic hormone when large amounts of unlabeled lactogenic hormone was added, suggest several possibilities. From Table 9 it will be seen that the major shift in activity is from the mitochondrial fraction to the supernatant fraction under these conditions. These changes amount at most, however, to a decrease of 35 percent in the activity of the mitochondrial fraction. It may be suggested that these results indicate that the particulate association is a reasonably firm one, particularly with the microsomes, since the exchange was not major. It would also appear that these results are suggestive evidence that the radioiodine label is remaining attached to the lactogenic hormone molecule and not being exchanged to other molecules in the cell. If such exchange were taking place the addition of untagged lactogenic hormone should have had no effect on the distribution of the I^{131} activity.

Turning now to a discussion of the results which have been obtained with the various chemical fractionation procedures the results of experiments utilizing trichloracetic acid, guanidine hydrochloride and streptomycin will be considered.

Since it was found that approximately 95 percent of the homogenate activity resides in the cytoplasmic fractions of the cell these studies were carried out on the "unfractionated particulate preparation" as previously defined. Three experiments (Table 10) show that at least 95 percent of the I^{131} activity of the cytoplasm from animals sacrificed one hour after the initial injection is associated with large molecules precipitable in 5 percent trichloracetic acid. This suggests that the activity is remaining in association with large protein molecules, undoubtedly the lactogenic hormone.

The original studies of Claude (1946) and Schneider (1946) suggest that practically all of the pentose-nucleic acid of the cytoplasm is found in

the particulate bodies of the cytoplasm. This being the case it was considered possible that the large degree of particulate localization might, in fact, represent association in some manner with the pentosenucleic acid or the pentosenucleoprotein of the cytoplasm.

The studies using methods specific for nucleoprotein precipitation (Table 10) indicate that this assumption was correct. These two specific methods, which gave no precipitation with free labeled lactogenic hormone, show that the majority of the activity is precipitable as nucleoprotein. The agreement between the results obtained with guanidine hydrochloride and those with streptomycin methods is good and indicates that at least three-fourths of the labeled hormone found in the cytoplasmic portions of the mammary gland homogenates is in a form which is precipitable as nucleoprotein. Qualitative chemical tests of the precipitated material indicate that it is indeed a pentosenucleoprotein and that the radioiodine activity resides in the protein portion of the precipitated material.

These findings—that the particulate localization of the labeled lactogenic hormone accounts for at least 80 percent of the activity present in the mammary gland homogenates and that the cytoplasmic material precipitable as nucleoprotein accounts for at least 75 percent of this activity—seem to substantiate one another. It may be suggested then that these two distribution-phenomenon are in reality the same. It might be inferred that 80 percent of the lactogenic hormone entering the mammary gland cell under these conditions becomes associated with the cell particulates, the mitochondria, and microsomes and that at least 75 percent of this hormone is associated with cytoplasmic pentosenucleoprotein, which is found predominantly in the particulates.

The attempts at solubilization of the guanidine hydrochloride precipitated "labeled lactogenic hormone-nucleoprotein complex" have been virtually unsuccessful. Results of these attempts are presented in Tables 11 and 12 where soluble non-dialyzable I^{131} activity has been used as the measure of material taken into solution. In later work this was extended to include precipitability by M/200 streptomycin. One can only conclude from these studies that this material is rather insoluble for the conditions employed or as measured by these criteria.

This insolubility was not too surprising in the light of the well known low solubility of the cytoplasmic particulates (Paigen, 1954). In the case of the precipitated nucleoprotein material it is possible that denaturation may help explain the low solubility. It can be suggested from these studies, however, that the association of the labeled lactogenic hormone with the cytoplasmic particulates and pentosenucleoprotein appears to be a rather stable one and that the particulate lipids do not appear to play a role in this association. It would be of interest to determine the effect of reducing agents on this binding.

The results of comparative studies of the loss of radioiodine from free labeled lactogenic hormone and from the "complex" taken into various solutions suggest several possibilities. The free radioiodine which is lost upon dialysis may have several sources in these preparations: labeled lactogenic hormone bound to the cellular pentosenucleoprotein; labeled lactogenic hormone adsorbed onto pentosenucleoprotein; free labeled lactogenic hormone; and, finally, the radioiodine has the possibility of having come from some other portion of the precipitated material if transfer of the label has occurred.

The data in Table 14 would seem to rule out the possibility of free labeled lactogenic hormone being the source of the I^{131} which is lost. It also does not appear likely that the activity could have come from labeled lactogenic hormone adsorbed on the pentosenucleoprotein as this iodine should be lost by dialysis to no greater extent than the label on the free lactogenic hormone. Two possible sources remain then for this radioiodine which is lost by dialysis; either the radioiodine label has not remained attached to the lactogenic hormone but has transferred to some portion of the pentosenucleoprotein where its energy of bonding is less or the labeled lactogenic hormone is bound to this pentosenucleoprotein and this binding has resulted in a change in the energy of bonding of the I^{131} to the lactogenic hormone molecule.

If the results obtained are considered in relation to the known facts concerning this type of iodination which has been carried out in the labeling of the lactogenic hormone, the possibility that exchange of the radioiodine has occurred to any great extent can be ruled out, thus bearing out the assumption that the labeled lactogenic hormone has become bound in some manner to the pentosenucleoprotein of the cell. The studies of Li, Lyons and Evans (1941) indicate that iodination of the lactogenic hormone takes place only by formation of diiodotyrosine groups. The iodine of diiodotyrosine is held by moderately high bonding energies and, as seen in the work reported here, the I^{131} label of the "labeled lactogenic hormone-nucleoprotein complex" can only be held by relatively low energy bonding. This being the case and since it is not likely that the I^{131} has been transferred from a high energy bonding to one of low energy, it can be assumed that the bonding energy of the I^{131} label on the lactogenic hormone molecule has been changed as a result of bonding between the lactogenic hormone and the nucleoprotein. This assumption fits all of the experimental data.

One other possibility exists, which does not affect the validity of the conclusion stated. This is that as a result of a decrease in the I^{131} to lactogenic hormone bonding energy, which may take place as a result of bonding between the lactogenic hormone and the nucleoprotein, exchange of the I^{131} label to some other molecule may occur. This, however, does not affect the

conclusion that the lactogenic hormone does actually become bound to the pentosenucleoprotein of the cytoplasm.

It is now possible to state several general conclusions which are indicated by the data. It may be concluded that the greater amount of labeled lactogenic hormone which enters the rabbit mammary gland cell becomes associated with the particulates of the cell, the mitochondria and the microsomes. Secondly, it may be concluded that this association with the cytoplasmic particulates is in reality a combination, probably of a chemical nature, with the pentosenucleoprotein of the cytoplasm. Accepting these two major conclusions, it will be of interest to consider the possible meaning of these findings in terms of the mode of action of the lactogenic hormone.

Implications

The importance of the cytoplasmic particulates, the mitochondria and microsomes, which are in large part made up of pentosenucleoproteins, has been reviewed with particular reference to the wide range of cellular metabolic reactions with which they are associated. Indirectly at least, the importance of the particulates and the cytoplasmic nucleoprotein has been discussed in relation to the functional activity of the mammary gland.

The major concern here is with a physiological process; that of the initiation of milk secretion. This apparently consists of two simultaneous processes; a shift in the cellular synthesis from the production of cellular material to the production of milk constituents, and an increase in the synthetic rate of the mammary gland cells. These synthetic processes, as has been seen, depend upon the metabolic reactions which are associated with the mitochondria and microsomes. It is possible to relate these changes in gross metabolism, that is, increased synthesis and/or shifted synthesis, in many different cases to changes in the amount of pentosenucleic acid, pentosenucleoprotein, or numbers of mitochondria in the cell.

To consider first the case of the mammary gland; certain changes in mitochondrial number and pentosenucleic acid content can be shown to occur in relation to the onset of lactation. The observations of Weatherford (1929), Jeffers (1935), and Dempsey *et al.* (1947 on the mitochondria have been described previously and indicate the presence of increased numbers of mitochondria in the secretory cells of the mammary gland during lactation. The studies of Kirkham and Turner (1935), also described previously, show the presence in the mammary gland of increased amounts of pentosenucleic acid during lactation. The level falls rapidly with the cessation of lactation. The findings of McShan *et al.* (1950) indicate that this increase is a result of lactogenic hormone action in the cell. It may be concluded, at any rate, that increases in cellular particulates and pentosenucleic acid are inexorably bound up in the process of milk secretion by the mammary gland cell.

Turning to other examples of increased cellular synthesis or shifted

cellular synthesis it will be seen that this phenomenon of an increase in mitochondria and in pentosenucleic acid goes hand in hand with such changes.

If other mammalian tissues are considered under conditions in which an increase in synthesis is observed, evidence of these same changes will be noted. Considering first the liver, since the largest number of studies have been carried out on this tissue, it is found that numerous studies have been carried out concerning the changes in pentosenucleic acid and in cytoplasmic particulates which accompany various changes in the liver metabolism. It has been found that fasting of experimental animals results in loss of cytoplasmic granules and PNA with no loss of DNA (Davidson, 1947). Drochmans (1947) found that ligation of the pedicle of one lobe of the liver in mice caused the disappearance of the basophilia of the cytoplasm (decrease in the number of microsomes) and a decrease in the PNA. It has been reported (Novikoff and Potter, 1948) that in regenerating liver after partial hepatectomy the PNA content is markedly increased, reaching a maximum at the time of most rapid growth. Lombardo *et al.* (1953) found an initial marked increase in the PNA content of regenerating liver which appeared to precede the subsequent growth. The hyperplastic liver tissue of rats fed 2-acetyl-aminofluorene has been shown by Pirozynski and Bertalanffy (1952) to contain increased PNA in the cytoplasm. These reports in the literature seem to show then that under many conditions of increased growth, and thus synthesis, in the liver, the PNA and the number of cytoplasmic granules are increased.

A miscellany of other mammalian tissues have been studied for changes in PNA content under various conditions. Drasher (1953) found that during pregnancy in the mouse the uterine and placental PNA increased. Logan (1952) in a study of nerve regeneration after nerve crush or section reported an increase in PNA followed by increased DNA and protein. In study of the effect of castration and testosterone on the rat seminal vesicle, it was found by Rabinovitch *et al.* (1951) that castration lowered the PNA/DNA ratio and that testosterone reversed this change. These same workers (Rabinovitch *et al.,* 1952) found that following duct ligation of the submaxillary glands of mice the total PNA decreased and the PNA/DNA ratio decreased. Growth in the thyroid gland has been correlated with a high PNA content of the gland by Reich (1949). It has been reported (Meyer-Arendt, 1952) that antibody production in the spleen of mice is accompanied by large increases in the cytoplasmic PNA.

Several other studies on non-mammalian forms have shown the same general pattern. Takata (1952) has reported that the cells of the regenerating lenses of adult *Tritutus pyrrhogaster* show an increased PNA content. Limb regeneration in tadpoles has been demonstrated by Barakina (1952) to be accompanied by more than normal amounts of PNA.

The findings which have been made in the field of developmental embryology also reflect the importance of the cytoplasmic particulates and the pentosenucleic acid in cellular synthetic processes. Gustafson and Lenicque (1952), in studies of the development of sea urchin eggs, demonstrated that the number of mitochondria increases markedly at the same period of development in which there is a marked rise in respiration and in certain enzymes, and during which the presence of new proteins can be demonstrated (Gustafson and Hasselberg, 1951; Perlmann and Gustafson, 1948). Steinert (1951), in a study of the synthesis of ribonucleic acid in the developing embryo found that the increase in ribonucleic acid followed the same pattern; that is, an accelerated increase in concentration beginning at the gastrula stage. Gustafson (1954) indicates that the increased synthesis of nucleic acid metabolites which occurs at this stage is related to the growth of mitochondria, possibly from microsome-like precursors. Hultin and Wessel (1952) noted that purine metabolism and pyrimidine metabolism are accelerated at this same period. It has also been demonstrated in embryo development that the areas of intense differentation, and presumably the highest synthetic rate, have the largest population of mitochondria and the greatest content of PNA (Brachet, 1941; Steinert, 1951).

Chantrenne (1947) and Jeener (1948) have suggested a theory of mitochondrial development which may have some bearing on the present problem. They found that the particulates of mouse liver could be fragmented into a series which varied in size, PNA percent, and enzyme content. From this finding the idea was proposed that in the cell these heterogeneous particulates represent various stages in the development of fully active mitochondria. Thus, the enzymatically inactive, small particulates with high PNA content (microsomes) are gradually built into mitochondria through the addition of various enzymes.

Recent investigations of this problem have taken the viewpoint that the heterogeneous population of particulates may represent different metabolic entities; however, much of this newer work might also be interpreted as indicating growth or development of small particulates into large fully active mitochondria through the addition of enzymes, co-enzymes, etc., (Paigen, 1954; Kuff and Schneider, 1954; Novikoff *et al.*, 1953). Eichenberger (1953) has made observations with the electron microscope which appear to show the development of microsomes into mitochondria in the kidney.

The investigations of Hultin (1950b, 1952, 1953) on the incorporation of N^{15} ammonia, glycine, and alanine, and C^{14} carbonate into various fractions of developing sea urchin eggs indicate an active uptake into microsomes at the same time that there is a highly active incorporation of these substrates into non-soluble protein, presumably of the microsomes. At a later stage, as the great increase in mitochondrial number takes place, the

rate of incorporation of these substrates into mitochondria equals the rate for the microsomes.

Two possible conclusions may be inferred from the studies which have been considered. For increased synthesis to occur in a cell it is likely that there must be an increase in the functional units of the cell, the particulate bodies and their component nucleic acid. While it cannot be considered more than a possibility that the mitochondria develop from microsomes, this concept seems necessarily true at least to the extent that the mitochondria, with all its components, must develop from some smaller precursors and the development must include the addition to their structure of various enzymes and co-enzymes.

The results of this investigation, when considered in the light of these concepts, suggest several broad possibilities for the action of the lactogenic hormone on the mammary gland secretory cell. Considered in this manner two possibilities are noted. The lactogenic hormone in its intimate attachment to the pentosenucleoprotein of the particulates may be exerting its influence in two ways; it may be affecting enzymes which are present in the structure of the particulates, or alternatively it may be preventing the attachment of certain enzymes or co-enzymes to the particulates in the course of particulate development.

If a new field of study is now examined, that of virus reproduction, some insight may be gained into the manner in which the synthetic processes of a cell may be shifted even though the synthetic rate remains the same. Again it will be seen that the mitochondria and the pentosenucleoproteins of the cell are involved. Putnam (1953) reviewed the rapidly increasing literature concerned with bacteriophage reproduction and summarized the important findings: Exogenous substrates must be assimilated by the cell during phage growth in the cell; phage infection is accompanied by a striking alteration in the direction of metabolism of the cell with phage nucleic acid and protein being synthesized at the expense of complete loss of synthesis of cellular protein or nucleic acid; no changes in enzymes have been noted (exception is the increased deoxyribonuclease) and the cell is unable to form adaptive enzymes; and finally, the respiratory rate and the RQ of the cells is not affected by phage infection. In relation to this latter point, Mudd (1952) has stated that the bacterial mitochondria and cytoplasmic nucleoprotein persist throughout phage growth in an infected cell in morphologically unaltered form.

Cohen (1953) has presented evidence which indicates that the phage reproduction proceeds in what is essentially an enucleated cell since the cell DNA is degraded to the level of nucleosides. Apparently then, the striking shift in metabolism is due to an effect of the infecting virus upon the cytoplasmic centers of synthesis, the mitochondria, and associated nucleoprotein.

It does not appear to be the case here that synthesis is directed by the development of new enzyme systems, but that the shift in synthesis must be brought about by a redirection of existing systems.

That this may be the case is indicated by the work of Kozloff (1952) in which he found that approximately 47 percent of the phage N^{15} entering the cell was associated with cellular debris and that up to 35 percent of the activity entering the cell which was not recovered as virus, was sedimented between 3000 and 18,000 times gravity. This may indicate particulate attachment.

Here then, exists a system in which there is a dramatic shift in synthesis; it is not a result of new enzyme systems but of a changed utilization of existing systems. Work has been cited which indicates that this shift in synthesis may involve the mitochondria and pentosenucleoprotein and may even involve the attachment of virus or parts of virus to these cytoplasmic particulates. One way in which such a shift in synthesis might be affected could be through a process of inhibition of one enzyme system so that substrate would be funneled into a different system for the synthesis of new material.

As a generalization, then, there are several possible ways in which the lactogenic hormone attachment to the cytoplasmic particulates and pentosenucleoprotein may be effective in directing the extensive changes in cellular synthesis and metabolic rate which occur at the onset of lactation. The hormone may be directing these activities by controlling the composition of the cytoplasmic particulates which are so vitally important to these activities; or the hormone may be acting on the complete particulate to direct the rate and extent of utilization of particular substrates in the variety of metabolic pathways which any single substrate may enter.

It seems evident that future investigations into the mode of action of the lactogenic hormone on the initiation of lactation will have to be concerned with answering the question of whether these proposals are, in fact, the manner in which the hormone carries out its activity. At least the place to look for the answer is now clear: the cytoplasmic particulates and pentosenucleoprotein.

SUMMARY

1. Indication was found that following intraductal injection of I^{131}-labeled lactogenic hormone into the rabbit mammary gland the maximum uptake into the tissue occurs in less than one hour.

2. Using methods of centrifugal fractionation of mammary gland homogenates, it was found that the great majority of intraductally injected I^{131}-labeled lactogenic hormone, probably three-fourths or more, becomes associated with the particulate bodies of the cytoplasm, the mitochondria, and microsomes.

3. Differences were shown in the distribution of I^{131} and I^{131}-labeled rabbit serum proteins when compared with the distribution of I^{131}-labeled lactogenic hormone in rabbit mammary gland cytological fractions.

4. Following addition of unlabeled lactogenic hormone to mammary gland homogenates from animals injected intraductally with I^{131}-labeled lactogenic hormone little shift in the distribution of the activity occurred.

5. More than 95 percent of the cytoplasmic I^{131} activity in the mammary gland following intraductal injection of I^{131}-labeled lactogenic hormone was precipitated in 5 percent trichloracetic acid.

6. Seventy-five percent or more of the cytoplasmic I^{131} activity in the mammary gland following intraductal injection of I^{131}- labeled lactogenic hormone was precipitated as nucleoprotein with 4 molar guanidine hydrochloride and molar/200 to molar/400 streptomycin.

7. The guanidine hydrochloride precipitated nucleoprotein was relatively insoluble in 0.14 molar sodium chloride, 50 percent ethanol, 0.0034 normal ammonium hydroxide, and isotonic sucrose.

8. Studies using 4 percent desoxycholate, excess n-butyl alcohol, and 1 molar potassium hydroxide for 12 hours at room temperature on the "unfractionated particulate preparation" indicated the stability of the I^{131}-labeled lactogenic hormone-nucleoprotein combination. These studies suggest that the particulate lipids are not involved in the association of the lactogenic hormone with the particulates.

9. Chloroform extraction studies also suggested the stability of the lactogenic hormone-nucleoprotein combination, with this treatment removing no more than one-third of the I^{131} activity from solution.

10. Dialysis studies conducted under various conditions suggested that the bonding energy of the I^{131} in the lactogenic hormone was changed when the lactogenic hormone became associated with the cytoplasmic particulate nucleoprotein. This suggests that chemical bonding between the lactogenic hormone and the nucleoprotein occurred.

11. The bearing that the results of this investigation might have on the problem of lactogenic hormone action on the biochemistry of the cell during the initiation and maintenance of lactation were discussed.

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