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In Vitro Metabolism of the Rat Mammary Gland

and

Observations on *In Vitro* Actions of Thyroid Hormones

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Report on Department of Dairy Husbandry
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INTRODUCTION

The mammary gland, the characteristic anatomical feature of mammals, has unique physiological attributes. Its ability to do biological work in terms of growth and secretion is remarkable. This transient activity terminates upon involution of the tissue (aging), the process being cyclic throughout the reproductive life of the female mammal. Growth, greatly stimulated by the state of pregnancy, supplies the morphological units (lobule-alveolar system) for secretory activity following parturition. It is reasonable to believe that each of the physiological states, growth, secretion, and involution, is characterized and mediated by a unique metabolic (biochemical) organization as well as a morphological one. These states are known to be under endocrine control through numerous hormones, some of which have not been clearly defined. Therefore, a single tissue within an adult animal undergos physiological cycles involving growth, secretion, and involution, each subject to rather close experimental modification, and in the case of the secretory state, essentially without cellular proliferation.

The product of this secretion is milk—a universal food for the newborn mammal and a fluid of considerable human nutritional and economic significance. It results from intense synthetic production of proteins, fats, and carbohydrate, each, in general, representing compounds unique from structural and protoplasmic materials of tissue and of blood. In spite of theoretical and economic importance of this tissue its biocatalytic spectrum and organization—mediators of the physiological activity—are almost unknown.

This work attempts to increase the understanding of the mammary metabolism on a cellular level, especially in comparison with various physiological states and the processes associated with it. The *in vitro* respiration, aerobic glycolysis, and respiratory quotient are studied as a basis for subsequent experiments. Then, study of adenosine triphosphate synthesis

and its modification by thyroid and other hormones is considered in terms of the mechanism of biological energy transformation in this tissue and as an example of endocrine modification of enzymatically controlled metabolism.

REVIEW OF LITERATURE

Control of Cellular Metabolism

Various tissues are functionally and morphologically different from one another. Upon the alteration of the physiological state of a tissue a correlation with a change in form is usually noted. But these observations are only manifestations of alterations in the tissue's metabolism—the translocation and intra-cellular catalyzed transformation of the organism's nutrients. The catalysts are called enzymes. Therefore, change in the function of tissue must be intimately associated with direct or indirect alteration of the cell's enzymes and it seems that morphology is only a secondary index of such changes. Whatever changes the form, the functional state or pathological state is, then, some agent which modifies directly or indirectly one or more biocatalytic units. Almost anything the organism may encounter may be such an agent either physiological (e.g., a hormone) or non-physiological [e.g., the drug, DNP (2, 4-dinitrophenol)].*

The protoplasmic system's catalytic machinery and its operation are determined by (1) the qualitative nature of the enzymes, i.e., by the exact spectrum of these specific catalysts, (2) their quantity, (3) their activity, and (4) their location, as has been discussed by Potter (1949).

The occurrence of an enzyme is believed to be determined by genes—one gene for one enzyme. Beadle's dramatic work with mutants of *Neurospora crassa* has been the basis for this concept (Beadle, 1946). This mold is able to grow in presence of a few simple compounds like inorganic salts, glucose, nitrate, and biotin which are all that are necessary for growth and its associated processes. Upon irradiation of the asexual spores and growing individual ones in a complex "complete" medium, it was learned that some of the organisms (mutants) then failed to grow when placed in the simple medium. By increasing the complexity of the simple medium through addition of various vitamins, amino acids, etc., a medium permitting growth could be defined. Its complexity was intermediate between that of the "complex" and "simple" environment. From many such mutations it became obvious that each one resulted in the destruction of one specific enzyme, including many which have an analogous activity in certain mammalian tis-

*Certain substances which are frequently referred to, both in this work and in the literature, are abbreviated according to the list at the back of the bulletin. These abbreviations are the same as those used by McElroy and Glass (1951, 1952).

sue. However, among higher animals there is little evidence which leads to a similar conclusion, although some support has been found for this concept in spite of the vastly more complicated organism. This complication is exemplified by the apparent extreme variation in enzyme occurrence from one tissue to another within the same organism.

Possibly imperfections in assay procedures prohibit the demonstration of extremely low levels of such enzymes and other factors to be discussed later merely limit the amount, activity, or localization in such ways as to mask their detection. Nevertheless, enzymes of common metabolic pathways seem to have almost universal distribution.

Studies with micro-organisms indicated that in face of a constant genetic constitution an alteration in the spectrum or pattern of apoenzymes may change. This has been interpreted to mean that "genes determine the potentiality of enzyme formation." Therefore, (Spiegelman, 1950) in some cases of lower life forms, it is impossible to identify the geneotype by the occurrence of enzymes. The extent and how the formation of "adaptive enzymes" is superimposed upon the one gene, one enzyme concept is yet to be defined for higher animals (Geschwind and Li, 1953). Control of metabolism by mutation, presumably an infrequent event resulting in a sudden deletion of an enzyme, is a drastic discontinuous process. Should the enzyme amount be controllable, the metabolic modification would be quite flexible and subtle. The enzyme content of yeast cells, which by all known criteria should possess the same genetic pattern, may be variable within wide limits. This adaptation phenomenon has led to the concept of self-duplicating, non-nuclear genes (plasmogenes) (Spiegelman, 1946) which are responsible for the formation of, if not identical with, the enzyme.

In the case of animals, the existence of a unique pattern of enzymes in each organ and even for the tissues within the organ is well established (Potter, 1947; Greenstein, 1947). A tissue's enzyme content has been observed to change considerably in short time intervals. This is illustrated by various enzyme changes in rat corpora lutea during the reproductive cycle (Meyer and McShan, 1950) and in brain and liver of newborn rats during the first few days of their life (Potter, *et al.,* 1945).

For the ordinary metabolic reactions to procede, the substrate and catalyst must have a certain spatial relation, the minimum distance of separation probably being some distance which is tremendously small compared to the cell. It is obvious that any discontinuity in enzymatic pattern or alteration of such possibility would potentially, profoundly alter the metabolism. Intracellular differentiation has been recognized throughout the history of histology and cytology (see De Robertis, *et al.,* 1948). Some of these morphological structures have been isolated in mass quantities from the intact

cell while presumably retaining a large measure of their intracellular properties. With these techniques, the concept of discontinuous intracellular enzyme distribution has been placed on a sound biochemical basis (Hogeboom, *et al.,* 1953; Schneider, 1953). Of the cytoplasmic units, the mitochondria represent the most dramatic localization of biochemical activity, which is so complex that it implies a complex structure or organization within the particle itself. Indeed, electron microscopy has demonstrated the existence of intramitochondrial structure (Palade, 1953). Biochemically, these particles are units of localized enzymes, many of which are organized into multi-enzyme systems. The system mediating the citric acid cycle (Krebs cycle or cyclophorase system) represents the key organization. Alteration of this localization may easily result in modification and control of the cell's net metabolism. It complicates the application of the concentration concept to the intact organism and even to isolated particulate systems.

An enzyme's *in vitro* activity (synonymous to concentration only under highly defined conditions) is altered by the concentration of hydrogen ions; other ions (ionic strength), substrates, products, cofactors, and inhibitors; and by temperature and the oxidation-reduction potential. It seems most reasonable to consider these factors as further agents for metabolic control and the hormones must be considered as members of this physico-chemical environment acting directly or indirectly to alter one or more of these factors.

By virtue of the occurrence of vast numbers of enzymes, it is becoming apparent that numerous substrates, which may also be products, may be able to undergo further reaction into many additional products. The reactions of acetic acid in animal tissues may be involved in formation of (1) acetyl-Co A (coenzyme A), (2) aceto-acetate, (3) acetylated aromatic amines, from choline, histamine, and glucosamine, (4) citric acid, (5) fatty acids, and (6) steroids (Lipmann, 1953). Such reactions are arrived at by methods which indicate only the presence of conditions that may permit the intact organism to mediate the reactions. The problem is whether the organism does—and to what extent and under what conditions —carry out one or more of these reactions. Non-Embdem-Meyerhof-Cori glycolytic metabolic routes have been clearly recognized (Dickens, 1953). Bloom, *et al.,* (1953) have shown that a non-glycolytic pathway accounted for more than 75 percent of carbon dioxide formed from glucose in the liver. However, the realization of the operation and the importance of alternate metabolic pathways are not new, e.g., considerable attention has been given to the Pasteur effect with partial success in explaining the mechanistic events (Johnson, 1941; also see Dickens, (1951). Potter (1944, 1947) has given attention to them as probable significant operations in pathological as well as physiological changes.

The qualitative and quantitative description of a specific cell's metabolism will undoubtedly depend on the enzyme's environment, localization, amount and of course on their specific qualitative nature (occurrence) with each factor interrelated with the others. It is as if each enzyme has information —information which feeds back up on another unit of metabolism, i.e., by circular causal effects.

Metabolism of the Mammary Gland

Milk secretion seems to arise chiefly within large, highly differentiated, cuboid cells which line the alveoli of the mammary gland. Such cells undergo little mitosis during a lactational period (Maeder, 1922; Jeefers, 1935; Speert, 1948; Reece and Warbritton, 1953). Furthermore, the content of RNA (ribonucleic acid) is extremely low in human, goat, and cow milk (0.25, 2.3 and 1.6 mg./100 ml., respectively) (Mandel and Bieth, 1948). These facts clearly fail to support the concept of rapid cellular turnover or actual extensive degeneration during lactation. The secreting cell must be considered a relatively stable structure. This secretion is a continuous process during a given period of lactation or at least potentially so and it derives all precursors from the blood, although many of them may be appropriately modified during the secretory processes. Also, materials from the blood serve as substrates for the tissue's maintenance.

Methods: The methods used to describe the transformation of blood materials into milk are the same as those used for the study of other tissues. Ideal conditions would involve the tissue remaining intact within the experimental animal and without the experimental procedure itself altering the processes. The latter condition is difficult, if not impossible, to achieve. Through observation of the same phenomenon by various procedures, the logical interpretation and interrelation of such data might circumvent the emperically indeterminate factors.

An obvious way to test the nature of an organ's blood-transported substrates is to study the composition of the blood as it enters and leaves the specific organ. Providing the collection of blood does not disturb the animal, such experiments are achieved under highly physiological conditions. Such arterio-venous (A-V) changes in blood composition have been studied only in large animals. The sample of arterial blood may be taken from any convenient vessel in the arterial system. The venous samples are taken just prior to the collection of arterial blood. The cows are very sensitive to the experimental procedure, resulting in rapid changes in the blood's composition. Indeed, this is so quick and extensive as to create serious objection to the validity of data. Graham, *et al.,* (1936) have used this technique (cow) by analyzing blood taken from the internal iliac artery by way of the rectal wall and the accessible subcutaneous abdominal vein. Venous

anastomoses are extensive and therefore blood taken from this source probably represents a good venous sample.

To avoid serious alteration of mammary blood flow and blood composition by the experimental procedure itself, the Missouri group has found that nembutal anaesthesia permits the continued normal rate and composition of milk secretion (Reineke, Williamson, and Turner, 1941; Reineke, Stonecipher, and Turner, 1941; Shaw, 1946).

One serious objection to data by the A-V method is the unmeasurable contribution by lymph. Not only is the rate of flow unknown, but its composition is ill-defined. Although its flow rate may be small relative to the blood flow, the rate with respect to milk may be significant. This is an unevaluated variable in all mammary A-V methods.

The perfusion technique is also especially applicable to the mammary gland of large animals. The methods and contributions of perfusion studies to lactational physiology have been reviewed by Silver (1952a) who regards the method as being essentially an *in vitro* one. Besides requiring considerable technical dexterity in preparation of the perfusion circuit, certain factors are a critical part of each isolated gland perfusion. Of these, vascular blocking is quite serious whether it be mechanical (fibrin formation) or chemical (vasoconstrictors). The latter substances are known to be present in shed blood, the usual perfusion fluid. In these methods, which repass a limited amount of perfusion fluid (blood) through the organ, the control of metabolite and "waste-product" levels, even should they all be known, poses a serious and nearly impossible problem. Should all these and other complications be accounted for, the criteria for viability have yet to be established and further, viability does not necessarily imply normal or *in situ* function. Certain factors, therefore, (difficult technique, only small numbers of preparations permissible, hence slow collection of data, the unknown degree of deviation from the *in situ* state and near absence of control data, limitation to a few species, restriction to the study of the lactating mammary gland, unknown significance of the disruption of the lymphatic drainage, and the extent of gland deterioration during the experiment) certainly place severe limitations on the application of this technique.

Application of radio isotope tracer methodology allows the use of highly normal intact tissue but also may be used in conjunction with *in vitro* experiments. Interest in this technique as applied to milk secretion was shown prior to the availability of nuclear reactor produced isotopes (Aten and Hevesy, 1938). Cyclotron produced Sr^{89} and Fe^{59} were also used in milk secretion studies prior to World War II (Ert and Pecher, 1940; Erf, 1941). Popjak, *et al.,* (1951) have effectively applied C^{14} techniques in the intact goat and perfused isolated bovine mammary gland (Cowie, *et al.,* 1951) to the mechanism of fat synthesis. Similar studies have recently been used

with mammary homogenate preparations (Popjak, 1953). With the inexpensive reactor produced isotopes, especially C^{14} and P^{32}, and the relative simplicity of their technical manipulation, this tool will no longer be neglected in milk secretion studies. The main difficulty resides in inherent complicated metabolism of any tissue which taxes extreme caution in data interpretation. When one technique suggests that processes are able to operate under a given experimental situation, isotope methodology might be expected to relate whether or not it does occur *in vivo.*

Tissue culture ordinarily is not considered a metabolic procedure in itself. But in the case of the mammary gland, extremely drastic alteration of this tissue is hormonally controlled by factors yet incompletely described. Furthermore, the cellular mechanism of such control is almost completely undefined. In this *in vitro* method where excellent control is possible, one factor or the interrelation of various factors (especially hormones) might be quite effectively clarified. With such highly defined preparations which are probably almost identical to the *in situ* material in many metabolic respects, it is reasonable to suspect that a fruitful approach to the problem of endocrine control and hormone-metabolic interrelations is being neglected. Successful culture of the mouse mammary gland has been reported (Hardy, 1950). Similar tissue from adult (pregnant) rabbits has also been cultured *in vitro* (Rozynek, 1948). Although tissue culture demands considerable skill and a mere trace of tissue is ordinarily obtained, it might at least be used as a source material for certain histochemical studies.

Histochemical techniques may indicate cellular (also, with less precision, intracellular or cytochemical) localization of certain chemical constitutents which may be associated with metabolism, e.g., certain enzymes and small molecule products and precursors. It might be considered a more refined histological approach. Most techniques have been under serious, often polemic, criticism (Danielli, 1946; Glick, *et al.,* 1951). These techniques actually embody many experimental procedures designed to describe the biochemistry of a single cell (or a very small number of them) (histochemistry) or of the various structures within a cell (cytochemistry). Glick (1949) considers the techniques to involve microscopic, chemical, or mechanical operations. Application of the microscopic methods to mammary gland tissue has been reported frequently (see Turner, Chapter 13, 1952). The mechanical isolation of intracellular constituents from mammary gland homogenates is yet to be reported. The procedure for this tissue requires the serious and meticulous efforts of many workers, since the task involves the use of several criteria of homogeneity and its degree without "alteration" from the intracellular state. The empirical establishment for any tissue of the necessary conditions makes this no light task. If it is not done, worthless data and confusion will result (Hogeboom, *et al.,* 1953). Should such pre-

parations be made, much might be learned concerning intracellular localization and differentiation of mammary gland function, especially in terms of those processes which are unique to the tissue. This would be true if a reasonable balance of the whole homogenate and sum of its parts would be demonstrated for each chemical entity studied. It is for these reasons that results from the study of intracellular localization of I^{131} labeled lactogenic hormone in the mammary gland (Williams and Turner, 1954) must be held in reserve.

Homogenates are more than starting material for particulate isolations. The entire mass of disrupted cells in suitable media has wide application. All that was present in the tissue remains in the homogenate, e.g., mitochondria, microsomes, secretory granules, nuclei, all the inorganic ions, coenzymes, carriers, and proteins. The characteristic of such preparations is the ability to dilute then until the endogenous oxygen consumption approaches zero. This is true for the activity of many enzymes which have an optimum level of a small diffusable molecule (Potter and Elvehjem, 1936). Upon addition of the specific cofactors and substrates for one specific enzyme (or system), its activity may be "isolated" and studied independently (Umbreit, *et al.,* 1949). Homogenates are the usual preparation for qualitative and quantitative evaluation of the specific enzymes present in a tissue but no insight into their actual *in vivo* operation is gained. They are tools for describing the parts of a whole. Their experimental utility is further advanced by their manipulative simplicity, giving a high degree of control and accuracy.

To learn the actual process of the whole requires the presence of the whole itself, intact and undisturbed by observations made upon it. With the possible exception of some "tracer techniques" and deductive thinking upon gross observations, this approaches an impossibility. Presumably the study of an intact cell (or group of them) *in vitro* would permit conclusions as to what the integrated cell might be able to do *in situ.* Such preparations would lack the simplicity of organization found in the homogenate but would begin to approach the *in vivo* condition with considerable increase in controllable conditions, technical simplicity and accuracy. This was the purpose of Warburg's (1926) development of the surviving thin tissue slice (Umbreit, *et al.,* 1949). As will be discussed later, the problem of achieving the thin slices is not at all easy for some tissues. The possibility of using whole cells is now reasonable in the case of liver, providing the method of isolation itself does not seriously alter them (Anderson, 1953). Any attempt to isolate free, intact mammary cells may prove to be extremely difficult. The first use of the thin surviving mammary gland slice seems to have been made by Grant (1935; 1936) for *in vitro* studies of lactose synthesis. The first use of this preparation for *in vitro* respiration studies (in the accepted sense) was made by Folley and French (1949).

The direct chemical examination of mammary gland tissue has been neglected. Although the information represents the static aspects of the tissue, it might be able to permit certain new deductions, especially when accompanied with more dynamic data. This is certainly true of small molecules such as phosphorylated and other metabolic intermediates and cofactors. The near absence of such data illustrates the general disinterest in this organ's metabolic functions. The description of the enzymic spectrum permitted by the homogenate technique has been given little attention. Since these procedures were developed for another tissue (generally liver) each enzyme assay condition must be empirically redefined—a relatively easy task following its original description. Data which have been collected for the glycogen content (Knodt and Petersen, 1946) and arginase (also alkaline phosphatase) activity (Folley and Greenbaum, 1947) (Fig. 1a) represents direct chemical examination of the mammary gland. Recent data by Kirkham and Turner (1953) (Fig. 2) on the RNA and DNA content of rat mammary glands in various physiological studies have shown important relationships between these compounds and the growth and function

The translation of a procedure developed for one tissue to a problem of mammary gland biochemistry may, in general, proceed with little difficulty. However, assumption of this is not warranted. This is especially true for enzyme activity assay in homogenates, particulate isolations, and possibly in certain chemical procedures. As will be demonstrated later, the biochemical knowledge of milk secretion in the mammary gland is quite limited compared to other organs like the liver and kidney. However, those studies reported to date have used a wide variety of techniques, some representing a combination of procedures. Early A-V methods which implied mammary gland fat synthesis from oxygen rich compounds (Reineke, Stonecipher, and Turner, 1941) have been confirmed with *in vitro* tissue slices (Folley and Funch, 1950), *in vivo* administration of radioactive precursors (Popjak, French, and Folley, 1951; Popjak, French, Hunter, and Martin, 1951), tracers through a perfused bovine udder (Cowie, *et al.,* 1951), and further, by the combined use of radio-acetate and homogenates (Popjak and Tietz, 1953).

Carbohydrates and Fats: In spite of its difficulties, the A-V technique may be said to indicate that the ruminant mammary gland consumes oxygen and releases carbon dioxide (Reineke, Stonecipher, and Turner, 1941; Shaw, 1946).

Kleiber, *et al.,* (1943) attempted to study rat mammary gland *in vitro* respiration on slices which in all probability failed to be suitable preparations. This position is strengthened by reports of much higher rates (Folley and French, 1948a, b; 1949 a, b, c). In spite of technical difficulties in preparing true thin slices and in selecting the base analysis for activity reference (Folley, 1949) it is certain that lactating mammary tissue respires, *in*

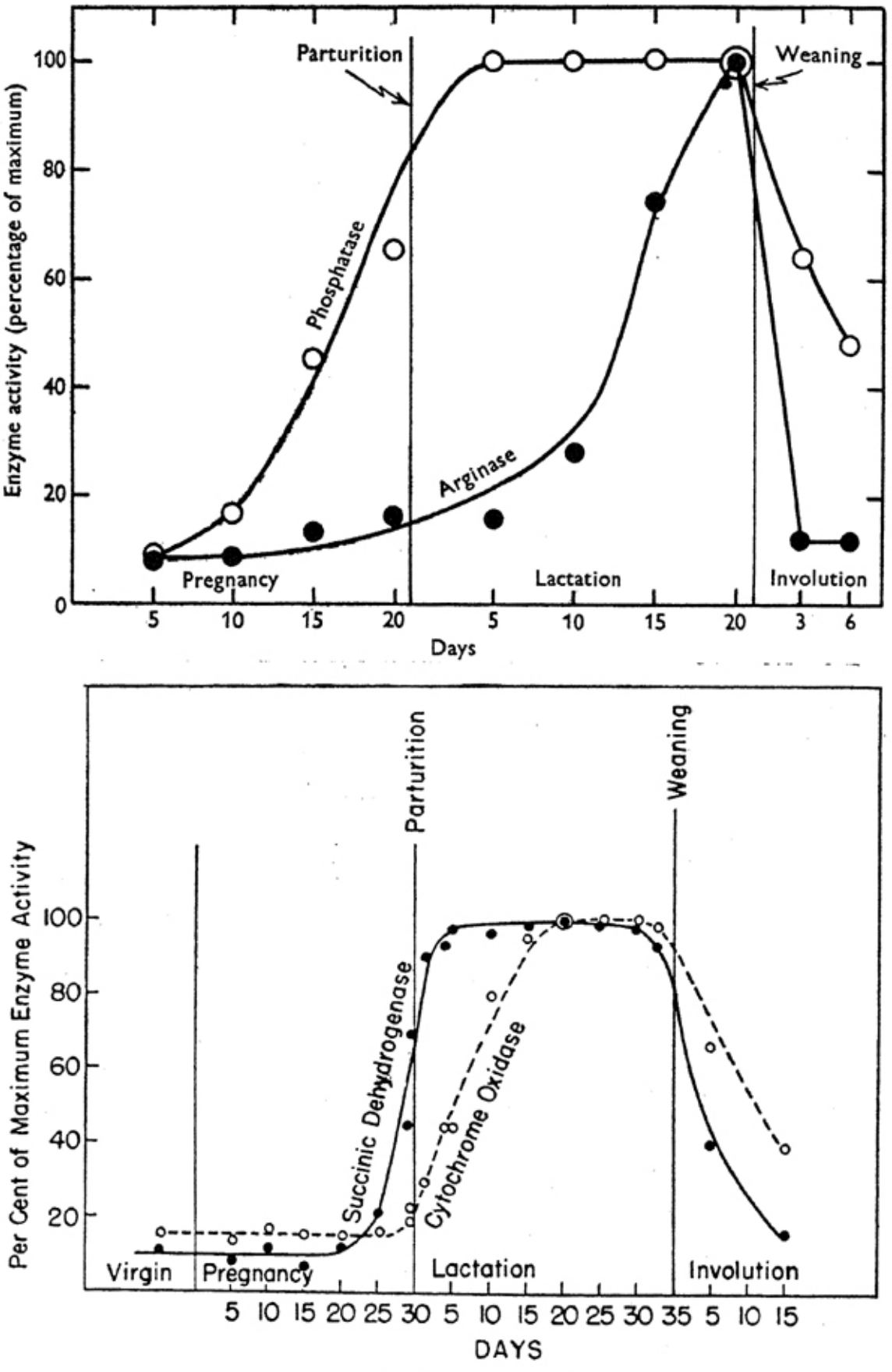

Fig. 1—Top: Alkaline phosphatase and arginase activity in rat mammay gland (Folley and Greenbaum, 1917). Bottom: Succinic dehydrogenase and cytochrome oxidase activity in rat mammary glands (Moore and Nelson, 1952).

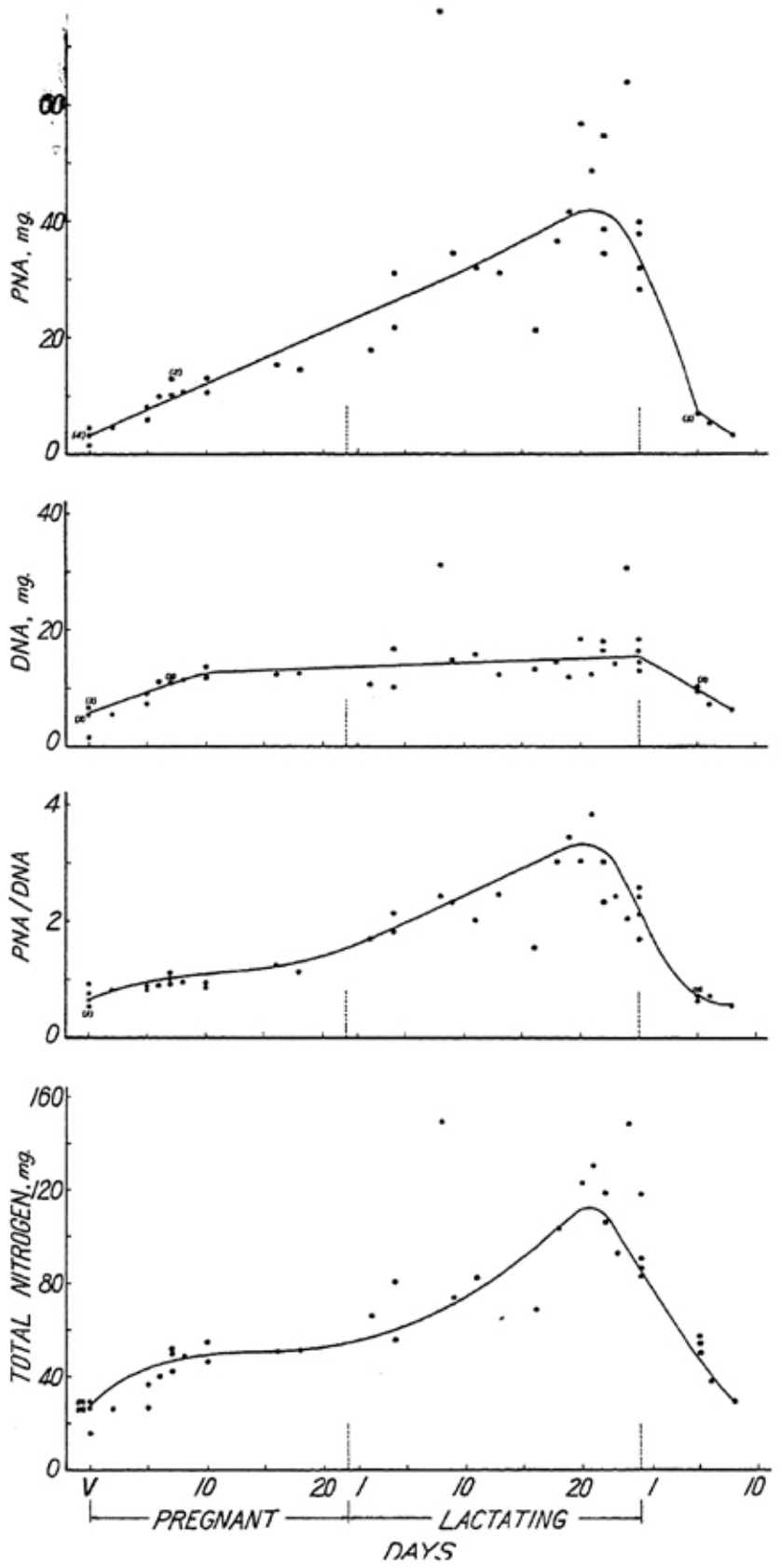

Fig. 2—Pentose nucleic acid, desoxyribonucleic acid, and nitrogen in rat mammary glands (Kirkham and Turner, 1953).

vitro, in presence of glucose at a rate comparable to other tissues. Rates of oxygen consumption (μl/mg. final dry wt./hr.) have been found to be about 10-15 for the lactating mouse; ca. 10 for the rat; 9 for the guinea pig; 5-6 for the rabbit; 4-5 for the goat; and 3-4 for the cow (Folley and French, 1949b, c). The mouse tissue compares favorably with rat liver but is lower than rat kidney. Lactating rat mammary glands respire in presence of mannose at a rate similar to rates in presence of glucose. D-galactose, D-fructose, L-sorbose, D-glucose-1-phosphate, N-acetylglucosamine, glucosamine, L-arabinose, D-arabinose, D-ribose, D-xylose, L-rhamnose, lactose, maltose, cellobiose, sucrose, raffinose, and glycogen failed to serve as metabolizable substrates (Folley and French, 1949b). However, G-1-P (D-glucose-1-phosphate) gave an R. Q. well above the other substrates which may only mean its partial transformation to glucose. The endogenous *in vitro* respiration of lactating rat mammary gland is high and especially in presence of substrates (glucose) retains constant rates over long periods (at least 3 hours). Endogenous respiration rates are relatively independent of the glands functional state. However, this activity in presence of glucose is highly related to the tissue's state with a great increase from the 20th day of pregnancy to 15-20 days lactation, which is followed by a decrease in activity upon removal of pups from the mother (tissue involution). Similar results are obtained when the data is calculated in terms of total abdominal mammary gland tissue (Folley and French, 1949c).

In presence of glucose, the R. Q. of tissue (mammary gland) is well above one in the case of non-ruminants but less than unity for tissue from the lactating cow and goat. The latter fails to support the concept of fat synthesis from oxygen rich sources which is implied in R. Q. measurements by A-V methods. Results of such A-V studies are tabulated by Folley (1949). In regard to the rat, the R. Q. is well below one at late pregnancy and near one by the first day of lactation. Involuted tissue is below one, which is also observed for tissue taken from any physiological state and measured in absence of glucose (Folley and French, 1949c).

Throughout late pregnancy, lactation, and even involution, the aerobic acid production of rat mammary gland increases (apparent glycolysis) but is always low. Apparent anaerobic glycolysis proceeds at considerable rates in such lactating tissue, indicating that, at least in this state, the Pasteur effect is marked (Folley and French, 1949b, c). It might be significant that the rate of apparent aerobic glycolysis is higher (although in absolute terms it is low) in involuted tissue than at other states while respiration undergos a decided drop.

It is well known that acetate is utilized by the mammal for fat synthesis (e.g., Bloch and Kramer, 1948) and, further, that ruminants produce large quantities of this acid through rumen fermentation (Elsden and Phillipson,

1948). Arterial blood fatty acid level is appreciable (Mc Clymont, 1949; Reid, 1950). Such information permitted Folley (1952a) to suggest that acetate could be the more direct oxygen rich fat precursor.

Indeed, mammary gland slices from sheep, goat, and cow consumed acetate *in vitro* (acetate the only exogenous substrate) with and R. Q. > 1; rat tissue failed to utilize the substrate and metabolized with R. Q. < 1; rabbit tissue was intermediate between these extremes. In the case of sheep the magnitude of this effect increased throughout the period of ca. 6 to 48 days *post partum*—the period of increasing milk yield. Glucose stimulates acetate utilization (glucose plus acetate substrate) in sheep, rabbit, and rat mammary slices. The effect upon the latter was trivial. Under such conditions rabbit tissue respired with R. Q. well above 1 and sheep tissue metabolized with an R. Q. greater than that observed with acetate alone (Folley and French, 1948a, b; 1949a, d; 1950). These observations are strong evidence that acetate is indeed a more direct oxygen rich milk fat precursor. Such *in vitro* data is consistent with A-V technique results and emphasizes the significance of a ruminant's unique alimentation.

Use of isotopic carbon has given confirmation to these generalizations concerning fat synthesis. A much larger fraction of rabbit milk fatty acids are derived from acetate than from carbohydrates. Degradation of specific fatty acids suggested the path of their synthesis was by way of glucose to pyruvate to acetate to fatty acids (Popjak, *et al.*, 1948; 1953). Studies on the fate of radio-acetate in the intact lactating cow is consistent with such a mechanism (Kleiber, *et al.*, 1952; 1953). Acetate is incorporated into various bovine milk constituents, but is dramatically utilized in fat synthesis (Table 1).

TABLE 1 -- RADIO--ACETATE INCORPORATION INTO MILK CONSTITUENTS (ADAPTED FROM KLEIBER, 1953)

Milk constituents	Amount of C^{14} in milk constituents in % of injected C^{14} acetate in 46 hrs. after injection	
	(carboxy C^{14}) acetate	(methyl C^{14}) acetate
Organic	16	17
Lactose	10	21
Casein	8	16
Fat	79	60

A Zilversmit (Zilversmit, *et al.*, 1943) product-precursor study of radio-acetate milk fat relationships in the intact lactating goat has shown that this two-carbon compound is rapidly utilized for milk fat synthesis and that plasma fatty acids could not be a major source of shorter-chain fatty acids. The same conclusions are given in milk cholesterol. Furthermore, the short-chain fatty acids of milk have a higher specific activity than the long ones—

an observation that implies the formation of higher fatty acids from lower ones (Popjak, *et al.,* 1950; 1951a). As a continuation of these experiments, each milk fat fatty acid was isolated. Upon measurement of specific activity of specific carbons in these pure acids, data were obtained which were consistent with the concept of stepwise two-carbon elongation of short chain acids to the longer ones. Possibly stearic and oleic acids are derived from non-acetate blood precursors (French, *et al.,* 1950; Popjak, *et al.,* 1951b). This early data on milk fat synthesis and some more general aspects of this problem have been reviewed by Popjak (1951). Similar data obtained by radio-precursor studies on the perfuse isolated bovine udder support data given by other technical procedures (Cowie, *et al.,* 1950; Cowie, *et al.,* 1951).

Ability of ruminant and non-ruminant mammary gland slices to use radio acetate in presence or absence of radio glucose has clarified intermediary metabolic differences of such species in presence of their substrates, as previously observed in R. Q. measurements. Balmain, *et al.,* (1952) confirmed the earlier observation that non-ruminant mammary tissues utilize glucose (R. Q. > 1) but the carboxyl carbon of acetate goes into fatty acids only in presence of glucose. In presence of glucose and acetate (both labeled) rat mammary gland slices use eight times as much glucose as similar preparations of ewe udder tissue, and one-sixth as much acetate. In the case of the rat, utilization of both substances for fat synthesis is about equal but for the sheep acetate is incorporaed 30 times as fast. Both species fail to discriminate to any large extent between the two acetate carbons (Balmain, *et al.,* 1954).

The present status of milk fat synthesis and its interrelation with carbohydrate metabolism has reached a high degree of elegance with Popjak and Tietz's (1953; 1954a) *in vitro* reconstituted cell free mammary gland preparations which are fully able to give net fat synthesis. These experiments combined radio isotope and rat (and sheep) mammary gland tissue homogenate techniques. Some data were obtained with tissue slices. Rat mammary gland slices failed to utilize acetate for fatty acid synthesis (confirmation of the work of Balmain, *et al.,* 1954). Pyruvate, OAA, α-ketoglutarate, succinate, and especially glucose increased the utilization of acetate (rat) and pyruvate (also members of the Krebs cycle) were almost as effective as glucose in this stimulatory role in the case of sheep. Fatty acid synthetic activity in rat mammary gland homogenates was dependent upon (1) presence of oxygen, although pure oxygen was inhibitory, (2) simultaneous oxidation of pyruvate, OAA, or α-ketoglutarate, and (3) presence of ATP (adenosine triphosphate) which inhibited similar preparations from sheep at a 0.01 M level. All saturated normal fatty acids with even numbers of carbons (C_6 to C_{18}) were synthesized. Sheep udder preparations showed

such activity with cosubstrate additions but OAA was able to stimulate it (Popjak and Tietz, 1954a).

Not only the whole homogenates but also the supernatants ("cell-sap") from homogenates centrifuged at high speeds (e.g., 104,000 g) (Popjak and Tietz, 1954b) mediate synthesis of fats. Such preparations as these are approaching enough simplicity to permit critical tests for direct hormone alteration of these metabolic processes.

The glycerol moiety of milk neutral fats has been postulated to be a limiting factor in their synthesis. This is reasonable in view of glucose's stimulation of fat synthesis by mammary gland slices (Folley and French, 1949d) and is given some support by the observation that radio-glucose imparts its label to glycerol *(in vitro)* as well as fatty acids of milk (rabbit and goat) (French and Popjak, 1951, Popjak, *et al.,* 1952). Furthermore, without increasing glucose uptake, glycerol increases both R. Q. and acetate utilization by rat mammary slices. Glycerol also produces some increased fat synthesis when sheep udder slices metabolize in acetate alone (Balmain and Folley, 1951).

Recent work shows that glycerol increases significantly, incorporation of acetate carbon into fatty acids (rat mammary gland slices) above the ability of glucose to effect these same events (Balmain, *et al.,* 1954). Rat and sheep mammary gland slices could not be shown to incorporate radio-acetate into glycerol *in vitro* (Balmain, *et al.,* 1952a, b). A Zilversmit study of milk glycerol and lactose following injection of radio-acetate (carboxy C^{14}) into a goat made reasonable the assumption that glucose from which lactose is rapidly formed is glycerol's precursor (French and Popjak, 1951; Popjak, *et al.,* 1952). A large portion (95%) of the C^{14} is in carbon one and three of glycerol. Further experiments (rabbits) using *in vivo* isotope techniques indicate that 65 to 95 percent of milk glycerol is newly formed in six hours, which implies that this substance is metabolized at a rate greater than fatty acids of the neutral milk fats (Popjak, *et al.,* 1953). In the study of glycogenic metabolism of biologically labeled glycerol (Popjak, *et al.,* 1952), it is known that it is incorporated asymmetrically into glucose whose radioactivity is restricted to carbons three and four (Schambye, *et al.,* 1954). Simultaneously Swick and Nakao (1954) obtained similar results. It seems that glycerol is probably formed in the mammary gland itself by mechanisms similar to those known to occur in microorganisms. It is a rapidly metabolized moiety of neutral fats which may be a limiting factor in their synthesis. Contrary to the previous discussion, the two-carbon fragment (acetate) plays a secondary role here and glucose assumes major importance.

Lactose is not metabolized by rat mammary gland slices *in vitro* (gives an R.Q. of 0.8.) nor does it increase endogenous respiration rates to the same extent as glucose (Folley and French, 1949b). The mechanism of lactose

synthesis, a unique function of the mammary gland, is still an unsolved problem of intermediary carbohydrate metabolism. Kaufman and Magne (1906) found that blood from the mammary vein (lactating cow) contained less sugar than jugular blood. Values were nearly equal in the dry cow.

Perfusion of sheep udders, which continued to secrete milk during the experiments, revealed similar information. Galactose was not used (Foa, 1912). It seems that the first conclusive *in vitro* (slices) demonstration of lactose synthesis was made by Grant (1935). Fructose, mannose, galactose were not suitable precursors. Galactose in presence of glucose was no more effective than the latter carbohydrate alone (Grant, 1936). Such observations were dependent upon the integrity of the cell and were non-existent in presence of low concentrations of fluoride and iodoacetate, which also prevented glucose utilization by the tissue slices. Certain phosphate esters were ineffective substrates. G-6-P (glucose-1-phosphate), F-6-P (fructose-6-phosphate), HDP (hexose diphosphate), galatose-6-phosphate and PGA (phosphoglyceric acid) (Grant, 1936), but such phosphates are generally found to be impermeable so they would not be expected to directly effect synthesis in intact cells. Use of an unspecific lactose analysis led Weinback (1936) to the conclusion that dehydrated lactating rat mammary glands were able to synthesize "lactose." Such broad over-generalizations illustrate the elementary stage of this problem (Folley, 1952). Although perfusion of the bovine udder with glucose containing blood increases tissue glycogen (Knodt and Petersen, 1949) which is decreased during formation of lactose and a similar preparation with insulin added to the perfusion fluid resulted in a decreased lactose synthesis accompanied with tissue glycogen deposition (Knodt and Petersen, 1946), it need not mean that glycogen is the immediate necessary precursor of the disaccharide.

It seems rather certain that glucose is the major source of both residues of milk lactose. *In vivo* use of C^{14}-glucose (rabbits) has led French, *et al.,* (1952) to this conclusion. Similar experiments involving the lactating goat confirm this (Barry, 1952; Reiss and Barry, 1953). Furthermore, these workers find that the glucose and galactose residues have nearly identical specific activities. By combined use of the perfused isolated bovine udder and (carboxy C^{14})acetate and C^{14} bicarbonate, it was learned that the former but not the latter was incorporated into milk lactose (Cowie, *et al.,* 1951), which indicates carbon dioxide fixation is not a pathway in lactose synthesis. Further experiments on such bovine udder preparations confirmed the equal incorporation of 1-C^{14} glucose into the glucose and galactose residues (Diamant, *et al.,* 1953). A Zilversmit analysis of incorporation of (carboxy-C^{14})acetate into lactose (*in vivo,* lactating goat) revealed that this substrate is a possible intermediate, with the "acetate" to hexose series of reactions being quite rapid (Popjak, *et al.,* 1952). Rabbit milk lactose from

animals given radio-intermediates (e.g. acetate and butyrate) was found to have a different distribution of radio-activity in the two residues and in such a way as to suggest that the galactose moiety may not be directly derived from the glucose chain (Schambye, *et al.*, 1953) (See Table 2 for role of other intermediates).

TABLE 2 -- CONTRIBUTION OF METABOLITES TO BOVINE LACTOSE SYNTHESIS*
(From Kleiber, 1953)

Intravenously injected Metabolite	Radioactivity in Lactose in % of activity in all organic milk constituents
Uniform C^{14} glucose	83
2-C^{14} Caproate	69
2-C^{14} Propionate	64
C^{14} Carbonate	59
1-C^{14} Propionate	56
1-C^{14} Valerate	49
3-C^{14} Norleucine	40
2-C^{14} Butyrate	38
C^{14} Formate	36
1-C^{14} Butyrate	34
2-C^{14} Acetate	21
1-C^{14} Acetate	10

*46 hours after injection

In vitro demonstration that lactating guinea pig mammary gland slices synthesize lactose, detected chromatographically from glucose and glycogen (Malpress and Morrison, 1950) leaves little doubt that this synthetic activity resides in the mammary gland itself. Maltose and lactate did not produce detectable quantities of this carbohydrate (also see Malpress, 1950; Malpress and Morrison, 1952; McGeown and Malpress, 1952).

Frozen homogenized lactating rat mammary tissue was able to phosphorylate galactose (also glucose, mannose, fructose, and maltose) upon addition of ATP (Crane, 1952, 1953). The known ability of other cells to convert galactose-1-phosphate to G-1-P in presence of uridine diphosphate glucose (Leloir, 1951) makes it reasonable to expect an enzymic explanation of lactose synthesis within the immediate future, especially in view of the fact that this galactowaldinase has been demonstrated to be present in the lactating rat mammary gland (Caputto and Trucco, 1952). Significance of neuramin-lactose, isolated from rat mammary gland (lactating), is unknown (Trucco and Caputto, 1954). Potter homogenates of lactating guinea pig mammary glands have been shown to occasionally synthesize lactose in presence of glucose, glycogen, or G-1-P (Reithel, *et al.*, 1952). Continuation of this work showed that a soluble protein fraction of mammary gland (unquestionably a very heterogeneous preparation) effected synthesis of lactose from G-1-P in presence of glycogen. Both conditions were without added magnesium, phosphate, and ATP (Kittinger and Reithel, 1953).

Anaerobic glycolytic degradation of glucose by mammary gland tissue (lactating rat) is extensive. Aerobic glycolytic acid production (apparent) is relatively low (lactating mouse, rat, guinea pig, rabbit, goat, and cow), i.e., the Pasteur effect is marked (Folley and French, 1949b). Extra energy requirements of lactation seem to be met by increased oxidation rather than glycolysis (Folley and French, 1949c). It is possible that even a large portion of apparent aerobic glycolysis may be a result of acid formation, e.g., citric and fatty acids, other than those of true glycolysis (Folley, 1949). Anaerobic glycolysis of mammary gland slices (lactating rat) was accelerated by nicotinamide (an inhibitor of Co I degradation) and accelerated further by traces of pyruvate (Terner, 1952). The protective ability of nicotinamide was not very marked in presence of rabbit mammary gland and in this case evidence for diphosphopyridine pyrophosphatase activity was obtained. Anaerobic glycolysis of lactating rabbit mammary slices was stimulated by HDP (in addition to presence of glucose, pyruvate, and Co I) and stimulated still more by further addition of yeast hexokinase. The hexokinase effect was also observed in mammary gland homogenates. Anaerobic glycolysis is uneffected by DNP. Rapid lactic acid formation was dependent on presence of Co I, pyruvate, and DNP, but glucose breakdown was not increased under these aerobic aconditions. In absence of DNP, $Q^{N_2}_{\text{Lactic Acid}}$ was greater than $Q^{O_2}_{\text{Lactic Acid}}$ without large differences in Q_{Glucose} between the two conditions being observed, i.e., increased lactic acid in absence of oxygen is not accompanied by equal glucose degradation (Terner, 1951a; 1952). The ability of mammary tissue to phosphorylate certain hexoses (Craine, 1952; 1953) and anaerobically produce acid seems to indicate the probable operation of the Embden-Meyerhof-Cori chain of reactions. Other data imply the operation of very active oxidative reactions involving glycolytic intermediates. The dehydrogenases of G-6-P, 6-P-G (6-phosphogluconic acid), and R-5-P (ribose-5-phosphate) show markedly large increases in activity during lactation (rat) (Table 3) (Glock and McLean, 1953, 1954). Also, with the ability to resynthesize hexose monophosphate from R-5-P, one suspects that less known oxidative (pre-citric acid cycle) metabolism is of considerable importance in mammary gland tissue (see Dickens, 1953), i.e., there may be alternative routes of metabolism.

TABLE 3 -- DEHYDROGENASE ACTIVITIES OF RAT MAMMARY GLAND
(From Glock and McLean, 1954)

	Substrate			
		6-P-G		
	G-6-P	pH 9.0	pH 7.6	R-5-P
20 days pregnant	86	92	50	197
5 days lactation	191	156	131	353
21 days lactation	5,452	1,734	883	1,300
2 days involution	50	93	45	147

Metabolic investigation of the transitory product of glycolysis, pyruvate, has been made on lactating mammary tissue *in vitro.* Trace quantities of fumarate are themselves without effect on oxidative rate but they strongly stimulate pyruvate oxidation and restore glucose breakdown in preparations depleted of endogenous substrates. Fumarate also reversed the oxidative inhibition of malonate—an observation which is predicted by the Krebs (citric acid) cycle theory. Knodt and Petersen's (1946) observation that endogenous substrates were metabolized in such a way that citric acid accumulated *(in vitro)* was confirmed, although the quantities were very small. DNP, a reagent which uncouples phosphorylation and therefore obliterates synthetic reactions, was found to increase oxygen consumption without simultaneous increase in pyruvate breakdown. This is indicative of a synthetic role (in part) of pyruvate. The effect of DNP upon oxygen consumption in presence of pyruvate was also observed in tissue from rats in late pregnancy. Glucose metabolism was not affected in a similar way (Terner, 1950, 1951b).

A full description of mammary gland oxidative metabolism is wanting. As previously noted, Terner (1951b) made some observations that imply the operation of the Krebs cycle. Succinic dehydrogenase and cytochrome oxidase activity has been measured in rabbit mammary gland tissue taken from virgin, pregnant and lactating animals, as well as from animals in which the tissue has involuted (Moore and Nelson, 1952) (Fig. 1b). Maximum activity of these enzymes was found during lactation. The values were less than 20% of their maximum when tissues was taken from virgin animals or from those in the first 20 days of pregnancy. Upon involution, the activity dropped to values approaching those of early pregnancy. The ratio of cytochrome oxidase to succinic dehydrogenase was reduced (with respect to the earlier physioloical states) in late pregnancy and early lactation. The reverse was true upon involution. In other words, the ratio of these activities is not constant throughout the functional cycle of the mammary gland. Furthermore, the two enzyme activities did not seem to change during the tissue's growth phase (first half of pregnancy) (Fig. 1b). The reserves of the cytochrome system have been observed to decrease during lactation (Tuba *et al.,* 1950).

A lactating guinea pig "cyclophorase" (R_3M) *in presence of* ATP and Mg^{++} mediated the following reactions: (1) OAA to citrate, in absence of pyruvate, (2) α-ketoglutarate and succinate to fumarate, and (3) α-ketoglutarate plus malonate to succinate. These reactions went only to the product indicated, not completely to carbon dioxide and water. Under these conditions fumarate absorbed only 4 μ atoms of oxygen, compared to a theoretical value of 30 if the product were OAA. Oxidation of citrate was greatly increased by nicotinamide, glutamate which is known to decrease

OAA's inhibition of malic dehydrogenase, Co I, and to some extent by cytochrome c. Only a very slow oxidation of citrate could be observed when cytochrome c, nicotinamide, Co II (coenzyme II), and Mn^{++} were added. While pyruvate alone was *not oxidized,* its presence increased the rate of OAA oxidation. A curious observation was the ability of the mammary "cyclophorase" to inhibit the highly active citrate oxidation of true kidney cyclophorase. This system ("cyclophorase" plus ATP and Mg^{++}), oxidizes fatty acids in presence of α-ketoglutarate in trace quantities ("sparking"). As expected from the inability of this mammary gland preparation to oxidize fumarate and malate, it was found that these compounds were unable to serve as sparkers. Sparker was necessary irrespective of the ATP level. Higher fatty acids, e.g., palmitate (C_{16}) and laurate (C_{12}), were difficult to oxidize and in some situations actually inhibited action upon low levels of α-ketoglutarate (Moore and Nelson, 1951a, 1952).

The theory of the Krebs citric acid cycle is based upon observations of pigeon breast muscle. No claim is made that it should predict metabolic operations in any other tissue or organism although it now seems that the processes are present in many biological preparations. Experimental evidence is as follows: (1) Rapid oxidation of the postulated intermediates, (2) Stimulation of respiration by trace amounts of these intermediates (3) Synthesis of citrate from OAA, and (4) Oxidative formation of succinate from fumarate or OAA (cf. Krebs, 1943). Isotope studies have given confirmation.

The preparation of cell-free, easily sedimentable, washed residues which require only molecular oxygen for the oxidation of pyruvic acid to carbon dioxide and water has been thoroughly described by Green, *et al.* (1948). It is termed the cyclophorase system for it is able to metabolize cyclically as predicted by the Krebs cycle. Exogenous additions of cofactors and usually even phosphate need not be added. Its oxidative processes were not limited to members of the Krebs cycle (e.g. proline, glutamic acids, and fatty acids). Oxidation can be coupled with ATP synthesis (Green, 1949). This insoluble residue behaves as if it were an organized, self-contained catalytic complex or system which emphasizes extensive sub-cellular organization. The criteria for the cyclophorase system is almost exclusively this integrated "physiological" activity. It would be presumptuous to claim that such a system from mammary gland tissue has been prepared and described, although certain observations suggest the operation of the Krebs cycle in the classical sense. However, even for the more classical system certain essential features are yet to be observed, e.g., the rapid oxidation of one of its members (citric acid).

Extensive auto-oxidation has been observed in lactating mammary gland homogenates (Moore and Nelson, 1951b).

Phosphorus Compounds: The biochemical role of phosphorus is so extensive and diverse as to place its importance to life along with oxygen, glucose, and water. Its well known presence in casein as an organic ester implies a special or unique activity of it in the mammany gland. But again, experimental foundations are too inadequate to permit more than a trivial description of the mammary gland's phosphorus metabolism.

Casein phosphorus is derived from plasma inorganic phosphorus (Aten and Hevsey, 1938; Sternberg, 1950; Saarinen, *et al.,* 1950; Colas, *et al.,* 1950; Simonnet and Sternberg, 1952; Barry, 1952) by an undefined pathway. Because it has been suggested that DNA (desoxyribonucleic acid) is proportional to a set of 2n chromosomes in a given organism and that RNA is intimately related to protein synthesis, these materials have been measured in rat mammary gland tissue taken from animals in various physiological states. DNA was found to increase during early pregnancy and it remained relatively constant throughout late pregnancy and lactation but tended to rapidly return to values (amount per 6 posterior glands) observed in virgin animals. RNA:DNA ratios increased from about 0.6 in virgin animals to ca 1.4 at termination of pregnancy. The maximum value (ca. 3.4) was reached at 21-22 days lactation and fell rapidly to low values upon involution (Kirkham and Turner, 1953) (Fig. 2). On the basis of nucleic acid phosphorus analysis, similar but less extensive observations have been made in the mouse (Albert, *et al.,* 1951), dog, and rat "in lactation" (Simonnet and Sternberg, 1952; Sternberg, 1950). In view of the fact that the pigeon crop gland is stimulated by pituitary lactogenic hormone, nucleic acid relations in this tissue are related to true mammary gland responses. Under lactogenic hormone stimulation, DNA increased for a few days (to a time of no further increase of mitotic figures) and then remained rather constant. The RNA: DNA ratio increased from 1.3 (unstimulated tissue) to 215 within five days. During this interval a similar increase in Q_{O_2}(succinate) was observed. Withdrawal of the hormone led to regression which was similar to that observed for involuted mammary gland tissue (McShan, *et al.,* 1950). Rate of incorporation of P^{32} into mammary gland DNA is lower than for RNA but this activity of both nucleic acids increased during pregnancy (Albert, *et al.,* 1951). The greater activity of RNA phosphorus was also observed in the lactating rat (Sternberg, 1950) and dog (and rat) (Simonnet and Sternberg, 1952). The concentration coefficient of mouse mammary gland "Phosphoprotein" reached a maximum about four hours after injection of P^{32} while the nucleic acids did not seem to have reached their maximum at 48 hours following injection (Albert, *et al.,* 1951). This was true for tissue taken from both pregnant and non-pregnant animals. It seems that nucleic acids are not the precursors of phosphoprotein phosphorus. Lactogenic hormone increased rate of inorganic phosphate turnover without altering its con-

centration in the pigeon crop gland. This hormone displayed the reverse phenomena in the case of organic acid-soluble phosphorus (Brown, *et al.*, 1951).

Evidence for presence of organic phosphates and their alternation in certain respects during functional changes of the mammary gland has been reported. Brenner (1932) showed that surviving mammary gland tissue effected autolytic breakdown of self contained organic phosphates. Phosphorus content of the mammary gland has been shown to increase during lactation, the increase being due largely to soluble organic phosphorus compounds, especially those more stable to hydrolysis (Barrenscheen and Alders, 1932). The ratio of organic phosphorus to total phosphorus from the udder of virgin cattle was reported to be higher than that of tissue from pregnant animals, still higher in the secreting gland, and the extreme (lowest ratio) was reflected by non-functioning glands from senile animals, i.e., organic phosphates apparently accumulated between lactation. An ATP-like compound was isolated from non-secreting tissue and dephosphorylation of AMP (adenosine monophosphate) was found to be affected more by lactating tissue than non-lactating material (Borst, 1932).

A KCl homogenate of lactating guinea pig mammary gland has been reported in a brief communication to respire with simultaneous uptake of inorganic phosphate. Only traces of lactic acid accumulated but 2 to 4 x 10^{-4} M p-nitrophenol inhibited this aerobic phosphosphorylation and stimulated production of lactic acid, i.e., inhibited the Pasteur effect. Anaerobic lactic acid production was extensive. Fluoride inhibited respiration but increased net phosphate uptake. Iodoacetate and arsenite also inhibited this oxidative phosphorylation (Terner, 1953). While this manuscript was in preparation these experiments on aerobic phosphorylation were reported in detail (Terner, 1954). Homogenates from lactating guinea pig mammary glands seem to have been used since preparation of such tissue from rats and rabbits had poor respiratory activity. P:O ratios of ca. 1.5 were observed when corrections were made for extensive phosphatase activity. The Pasteur effect was so pronounced that it was observed even after addition of rabbit muscle preparations which presumably represented considerable extra glycolytic activity in the final reaction mixture. This is the first non-cellular animal preparation to possess the Pasteur effect. The effect was more extensive than Meyerhof and Fiala (1950) observed in preparations of dried yeast. Oxidative phosphorylation and Pasteur reaction were inhibited in both preparations by p-nitrophenol.

Phosphatases, catalysts of unknown utility to the organism, have been shown to be highly active in mammary gland tissue and to an extent highly dependent upon the physiological state of this tissue. Alkaline phosphatase (rat) is low during early pregnancy but starts to continously increase by mid-

pregnancy to its maximum and constant activity at 5 through 20 days lactation. Activity fell moderately (to ca. 50% of maximum value) during involution (6 days post-lactation) (Folley, 1949) (Fig. 1a).

Nitrogen Compounds: Early work using primarily the A-V technique resulted in the conclusion that milk protein was derived from blood proteins, since the quantity of free amino acids absorbed by the mammary gland was too small to account for excreted nitrogen (Graham, *et al.,* 1938).

Application of radioisotopes is beginning to alter the concepts of milk protein synthesis. Campbell and Work (1952), using the rabbit, and Barry (1952), the goat (intact animal and perfused udder), have shown, following addition of labeled amino acids to blood *(in vivo)* or blood perfusate, that radioactivity of milk protein is higher than blood or perfusate protein activity. This indicates that blood protein cannot be a major source of milk protein amino acids. Intravenously injected S^{35}-methionine (lactating goat) also showed much greater activity in milk protein than in plasma protein. Furthermore, when the animal was in a fasted condition, the blood and milk relations were similar, although the maximum activity of the milk protein was reached 4½ hours following injection compared to 1¾ hours in the case of non-fasted conditions (Askonas and Campbell, 1953). This is in sharp disagreement with Reineke, *et al.,* (1941) who found by the A-V technique that fasting resulted in no amino acid uptake by the udder in spite of continued milk secretion.

The exclusion of the possibility that peptides are milk protein precursors has been given experimental support. Activity of amino acids in casein formed from injected radioactive amino acids was found to be independent of their intramolecular location, i.e., activity of these amino acids in peptides isolated from partial hydrolysates was the same as that from acids obtained from totally hydrolyzed protein. Valine seemed to be an exception (Askonas, *et al.,* 1954). Antibodies seem to be passed directly from the blood plasma to milk (rabbit) (Campbell, *et al.,* 1953; Askonas, *et al.,* 1954). Therefore, it seems that application of more decisive techniques makes untenable the postulation (Reineke, *et al.,* 1941) that non-amino acid materials are prominent milk protein precursors.

In certain species (rat, mouse, and guinea pig, in order of decreasing activity; trivial activity in rabbit, goat, and cow) arginase activity has been demonstrated. Although arginase is rather high in lactating rat and, to a lesser extent, in mouse mammary glands, levels in such tissue from other lactating animals (rabbit, cow, goat, and guinea pig) were found to be no more than 7 percent of the activity observed in the rat. Therefore, it seems that arginase need not be a necessary mediator or modifier of protein precursors in milk synthesis. In the case of the rat, its activity is low but tends to slightly increase during pregnancy and continues this slow increase through

about the first 10 days of lactation, at which time it increases rapidly and continuously to a maximum value at 20 days of lactation. At three days postlactation the activity had returned to the low levels of early and mid-pregnancy (Folley, 1949) (Fig. 1a).

Lactating rat mammary tissue was found to inactivate the lactogenic hormone *in vitro* to a greater extent than such tissue from pregnant animals (Meiter and Sgouris, 1953). Various enzymes and metabolic factors have been found in mammary tissue of one or more species. In most cases these have not been thoroughly studied nor found to have any obvious significance, e.g., the highest tissue (guinea pig) diamine oxidase activity was found in the mammary gland. Table 4 lists some of these enzymes (see Table 3 and Fig. 3).

TABLE 4 -- MISCELLANEOUS ENZYMES IN MAMMARY GLAND TISSUE

Enzyme	Reference
Glutamic dehydrogenase	Greenbaum and Greenwood, 1954 (Fig. 3c)
Catalase	Greenstein, et al., 1941
Xanthine dehydrogenase	Greenstein, et al., 1941
Purine oxidases	Michlin and Ryzoua, 1934
Diamine oxidase	Moore and Nelson, 1951c
Cytochrome c	Rosenthal and Drabkin, 1943
Acid phosphatase	Greenstein, 1942
	Dempsey, et al., 1947
ATPase	Craine, 1952
	Kirkham, 1954
Phosphokinases (various)	Craine, 1952
Diphosphopyridine pyrophosphatase	Terner, 1952
Lipases	Lovehart, 1902
	Virtanen, 1924
	Tateyama, 1925
	Kelly, 1938; 1943; 1948
Amylases	Grimmer, 1913
	Tateyama, 1925
Maltase	Kleiner and Tauber, 1932
Cathepsin	Greenbaum and Greenwood, 1954 (Fig. 3a)
β-Glucuronidase	Fishman and Anlyan, 1947
	Greenbaum and Greenwood, 1954 (Fig. 3b)
"spreading factor"*	Elliott and Turner, 1950
Transaminase (glutamic-aspartic)	Greenbaum and Greenwood, 1954 (Fig. 3d)
	Kirkham, 1954

* Enzymic nature has not been demonstrated.

Miscellaneous

Direct Hormonal Alteration of Mammary Gland Metabolism: The previous discussion of this tissue metabolism was essentially one that constantly implied mechanisms of hormonal control. However, more direct consideration to the gland's metabolic-endocrine interrelations has

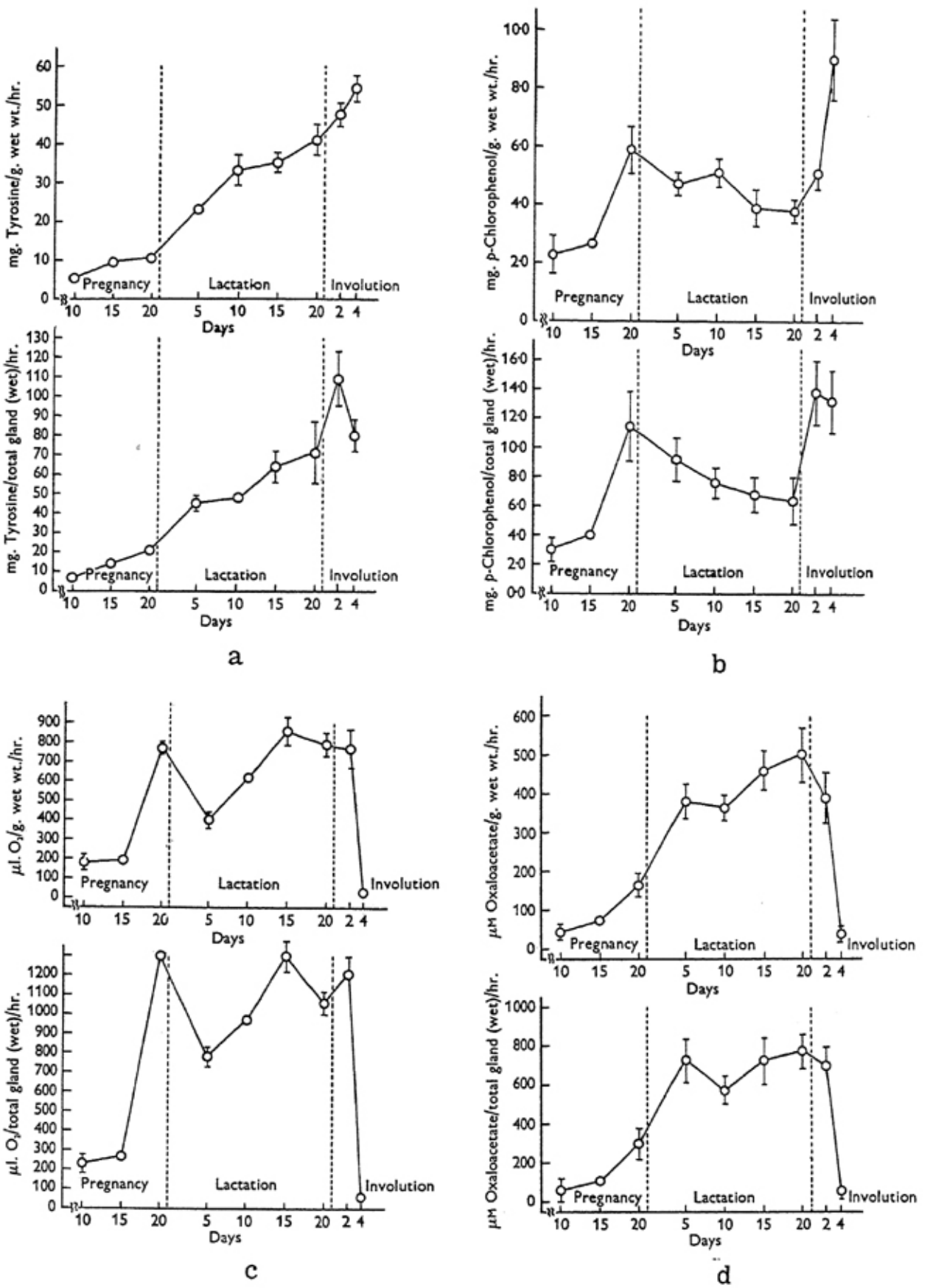

Fig. 3—Enzyme activities in rat mammary glands. (a) Cathepsin; (b) B-glucuronidase; (c) glutamic dehydrogenase; (d) glutamicaspartic transaminase (Greenbaum and Greenwood, 1954).

appeared largely as a tool to facilate further understanding of its functional dependence upon a complex of hormones which is yet to be fully defined. These studies have been reviewed by Folley (1952 a, b).

Although at least part of the biochemical structure responsible for fat synthesis is present in this tissue at late pregnancy (Popjak, *et al.*, 1949; Popjak and Beeckmans, 1950), the *in vitro* lipogenic effect could not be demonstrated upon tissue in this state (or following involution) in contrast to such demonstration upon lactating tissue. Presumably this effect is dependent upon action of galactopoietic and lactogenic hormones on the tissue and therefore may be an additional tool for further characterization of these factors. Since ruminant mammary gland tissue does not respond to this *in vitro* effect, it may mean that its action is closely related to carbohydrate rather than acetate metabolism. Glycerol's ability to mimic insulin in certain respects could be evidence that this hormone is concerned with processes which produce this alcohol. Insulin's lipogenic effects are antagonized by cortisone.

Net *in vitro* gas exchange (oxygen consumed plus carbon dioxide released) was increased in the presence of lactogenic hormone in the case of mammary gland tissue from lactating rats, i.e., as if R. Q. were increased. As was true for insulin, no effect was observed with tissue taken in late pregnancy. Such tissue, however, showed a greater gas net release in presence of cortisone.

Although arginase activity is dependent upon the physiological state (increased during lactation) and its activity is decreased following adrenalectomy, the fact that this enzyme has high activity only in the mammary gland of the mouse and rat makes it unreasonable to believe that it has an obligatory role in milk synthesis (Table 5).

TABLE 5 -- ACTION OF HORMONES UPON THE MAMMARY GLAND
(From Folley, 1952b, c)

Mammary Gland Preparation	Physiological State	In vitro Hormone Modifier	Hormone effect
rat; slices	late pregnancy and involution	insulin	none observed
rat; slices	mid-lactation	insulin (or glycerol)	increased R. Q., oxygen consumption and acetate uptake
ruminants	lactation	insulin	none observed
rat; slices	various	insulin plus cortisone	this steroid strongly depresses insulin's lipogenic effect
rat; slices	late pregnancy	lactogenic	none observed
rat; slices	early lactation	lactogenic	increased net gas release as if R. Q. were increased
rat; slices	various	cortisone	decreased incorporation of radioacetate into fatty acids
rat; slices	lactation	cortisone	decreases incorporation of radioglucose carbon into fatty acids
rat; homogenate	lactation; adrenalectomized		arginase activity decreased

Mechanism of Thyroid Hormones' Action. The well established fact that thyroxine is galactopoietic (Graham, 1934a, b; Blaxter, *et al.,* 1949) increases the desirablity of knowledge concerning its mechanism of action in the mammary gland —a mechanism which may well be identical to that in other tissues. Although this problem has been extensively studied in terms of thyroxine (see review by Barker, 1951), the recent demonstration that 3, 5, 3' triidothyronine more nearly conforms to the true thyroid hormone (Gross and Pitt-Rivers, 1953), places the problem in terms of this substance rather than thyroxine.

Characteristics of this new iodinated amino acid which earlier had been postulated to be a constituent of iodinated protein (Hird and Trikojous, 1948) include (1) much more activity than thyroxine in preventing thiouracil-induced goiter (rats), (2) ability to replace thyroxine in treating myxedematous patients, and (3) its normal presence in blood and thyroid tissue.

Barker in 1951 stated that "direct involvement of thyroxine in an enzyme system has never been demonstrated . . . . . ," neither has its *in vitro* presence stimulated tissue respiration as observed in tissue from animals in a state of hyperthyroidism (decreased in hypothyroid animals). Neither has an *in vitro* effect upon whole cell preparations "been conclusively demonstrated" (Dutoit, 1952). Tissue from hyperthyroid animals has consistently displayed higher metabolic activities, especially oxidative processes, than tissue from normal or hyporthyroid animals, i.e., no specific thyroxine-metabolic interrelation has been clearly recognized. This may mean the hormone's involvement in some reaction(s) common to many metabolic activities such as synthesis of enzymes, some of which are known to increase under the influence of thyroxine, e.g. cytochrome c (Drabkin, 1950) or biological energy yielding reactions, i.e., ATP synthesis (Dutoit, 1952). Such a view is made more plausible since discovery that DNP, a compound not too unrelated to thyroxine and which increases an animal's oxygen consumption, uncouples oxidative phosphorylation (Loomis and Lipman, 1948). Although in terms of metabolic influence of intact animals thyroxine and DNP are synergistic (Barker, 1946). There is no evidence that the latter is able to replace thyroxine in any of its physiological actions (Dutoit, 1952).

Synthesis of ATP, in higher animals, may be obtained by the action of (1) 3-phosphoglycerate kinase which mediates oxidation of 3-phosphoglyceraldehyde in presence of Co I with simultaneous formation of ATP, (2) pyruvate kinase upon phosphopyruvate to form pyruvate and ATP (Colowick, 1951), and (3) oxidative processes upon various members of the Krebs citric acid cycle (pyruvate, isocitrate, and α-ketoglutarate, succinate, and malate) giving a maximum number of high energy phosphate bonds of about 17 for complete pyruvate oxidation (Kaplan, 1951).

For over a decade oxidative processes were known to be closely related to maintance of ATP levels, e.g., in red blood cells and synthesis from the previously mentioned substrates. Intact cells were unnecessary. The activity is largely restricted to mitochondria. Such preparations of washed homogenate residues actively respire and take up inorganic phosphate when fortified with hexokinase, a soluble enzyme readily removed by washing, and the appropriate small molecules. Such a trapping system was used earlier in whole homogenate work (Belitzer and Goloskava, 1940). Details of oxidative phosphorylation of α-ketoglutarate have been defined in terms of purified proteins to occur as follows (Kaufman, *et al.,* 1953; Hift, *et al.,* 1953):

$$\alpha\text{-ketoglutarate} + \text{CoASH} + \text{DPN}^+ \longrightarrow \text{succinyl-SCoA} + CO_2 + \text{DPNH} + H^+$$

$$\text{succinyl - SCoA} + \text{ADP} + \text{Pi} \rightleftharpoons \text{succinate} + \text{CoASH} + \text{ATP}$$

which has the sum of:

$$\alpha\text{-ketoglutarate} + \text{DPN}^+ + \text{ADP} + \text{Pi} \xrightarrow{(\text{CoA})} \text{succinate} + CO_2 + \text{DPNH} + H^+ + \text{ATP}$$

P:O ratios (μ moles phosphorus taken up: μ atoms oxygen consumed) are usually used to express experimental oxidative phosphorylation results. Ochoa (1944) concluded that this ratio was 3 for oxidation of pyruvate and α-ketoglutarate, which has been confirmed with a more refined technical procedure by Slater and Holton (1954) although with lower values than reported by others, e.g., by Copenhaver and Lardy (1952).

Other pyrophosphates, e.g., uridine triphosphate, may become equally or more significant than ATP in the near future (Kalckar, 1954; Schmitz, *et al.,* 1954).

Thyroid-treated animals have a greater turnover of organic phosphates (Fraenkel-Conrat and Li, 1949; Venkataraman, *et al.,* 1950). However, a system of fresh mitochondria, the seat of oxidative activity, having the entire Krebs cycle intact were not found to be influenced by thyroxine as indicated by P:O ratios. When such measurements on similar systems were made upon tissue from hyperthroid animals, the P:O ratio was indeed decreased and rate of oxidation increased. The latter observation has been reported repeatedly. These observations (Lardy and Feldott, 1951) agree with these authors' hypothesis that the hormone acts by decreasing the net energetic efficiency of metabolism through uncoupling oxidative phosphorylation with, however, the higher oxidative rates yielding greater net amounts of available energy (ATP) during a time interval. Further work by these authors revealed that rat kidney mitochondria, malonate inhibited, had decreased efficiency of phosphorylation in presence of thyroxine at 10^{-5} M when metabolizing glutamate. Oxygen consumption was also depressed. This

effect could not be observed with mitochondria (liver or kidney) oxidizing other members of the Krebs cycle. Triiodothyronine acted like thyroxine except it did not decrease oxygen consumption (Maley and Lardy, 1953). Others have observed uncoupled oxidative phosphorylation in mitochondria from hyperthyroid animals (Martius and Hess, 1951; Hoch and Lipmann, 1953).

Only with a preincubation of liver mitochondria (Syrian hamsters, but not with rats) with thyroxine at low temperatures (presumably without buffer or substrate) has uncoupling been observed in Lehninger's (1949) system (Martius and Hess, 1951) and preliminary results with triiodothyronine indicated actions similar to thyroxine (Hoch and Lipmann, 1953). A brief note has indicated that triiodothyronine *(in vitro)* was unable to increase respiration of hemidiaphragms or liver slices from normal rats. By replacement therapy of hypothyroid rats, this compound was more effective than thyroxine in increasing oxygen consumption of tissues. In addition, respiration of a "rat heart homogenate-cytochrome c system with succinate as substrate. . . . was augmented by direct addition. . . . of either l-thyroxine or l-triidothyronine." In this preparation triiodothyronine showed that the "rate of oxygen uptake increased more rapidly, reached a higher point, and returned to the control level faster" than was observed with thyroxine (Wiswell and Asper, 1953).

EXPERIMENTAL RESULTS

In vitro Respiration, Aerobic Glycolysis, and Carbon Dioxide Production of Rat Mammary Gland Tissue.

In initiating studies on the metabolism and the role of the various enzyme systems in the growth and secretory activity of the mammary gland, it seemed desirable to follow the changes in respiration, aerobic glycolysis, and "metabolic" carbon dioxide production on true tissue slices *in vitro* throughout the entire normal reproductive cycle of the albino rat. From these data it should be possible to select metabolic reference points of maximum biological activities with reference to growth, secretory activity, and subsequent involution in the normal cycle which would serve as indices of the production of comparable biological activity under experimental conditions involving one or more hormones.

The complexity of the tissue with its variable gross chemical composition throughout the physiological cycle demands judicious selection of the activity reference base, especially when one wishes to compare the various physiological states. Activity is expressed as units/hour/mg./nitrogen in these experiments. Special care was taken to minimize the retention of colostrum and milk. At least, the nitrogen reference base minimized the effect of varying proportions of fat. With data on the nucleic acids of this tissue,

it has become more reasonable to use desoxyribonucleic acid as the activity reference base (Kirkham and Turner, 1953).

Methods and Materials. Virgin albino rats not in estrous and weighing between 150 to 170 gm. supplied the mammary tissue (inguinal and abdominal glands only) for the virgin state. Similar animals were bred to supply the glands in various stages of pregnancy and lactation. Only animals nursing more than five pups were used for the latter series. Involuted glands were obtained by removing 24-day old pups from their mother. The mother was sacrificed five days later. Most data were obtained by the method of Summerson (1939) with 0.3 percent glucose as substrate in at least duplicate, except for the virgins where the quantity of tissue was limited. About one-third of the data were obtained by the method of Dixon and Keilin (Dixon, 1951). Since these measurements were at random throughout the various physiological conditions and since both methods seem to agree quite well, these data were combined without designation. The gas phase was 95 percent O_2 and 5 percent CO_2; temperature 38°C; initial pH 7.2. All tissues were sliced with the Stadie-Riggs microtome (Stadie and Riggs, 1944). Slices were shaken in cold Ringer-bicarbonate medium, blotted between filter paper, again shaken in fresh medium, and gently blotted just prior to placing in the reaction flask. By use of true tissue slices, repeated leaching, and gentle blotting, it was hoped that the accumulation of colostrum in late pregnancy and milk throughout lactation was removed (Malpress and Morrison, 1950). A chemical test for reducing sugars in a saline medium containing slices was, following shaking at 38°C for 1 hour, only slightly but equally positive for tissue in mid-pregnancy, 20-21 days pregnant, and 13 days lactation. By using this standard procedure it is believed that these data for the various physiological states may be fairly comparable and that litle preformed milk leached from the slices during the experimental period.

Slices were removed from the metabolism flasks for colorimetric nitrogen determination at the end of the experiment (Umbreit, *et al.,* 1949). In all experiments the slices were allowed to metabolize for 30 minutes, which resulted in an O_2 consumption in excess of 50 μl (80 to 150 μl in most cases). The medium's pH did not decrease below 6.8 for the most active preparations during this interval.

Each point in Figs. 4 and 5 represents the average of two or three measurements on tissue from one animal, except those from virgins which represent one determination from one animal. The R. Q. was calculated from these averages. Data from an animal were discarded if the Q O_2(N) did not agree within 10 percent.

Results. Since the R. Q. is independent of variable gross tissue composition, more dependence may be placed on R. Q. values. Slices from vir-

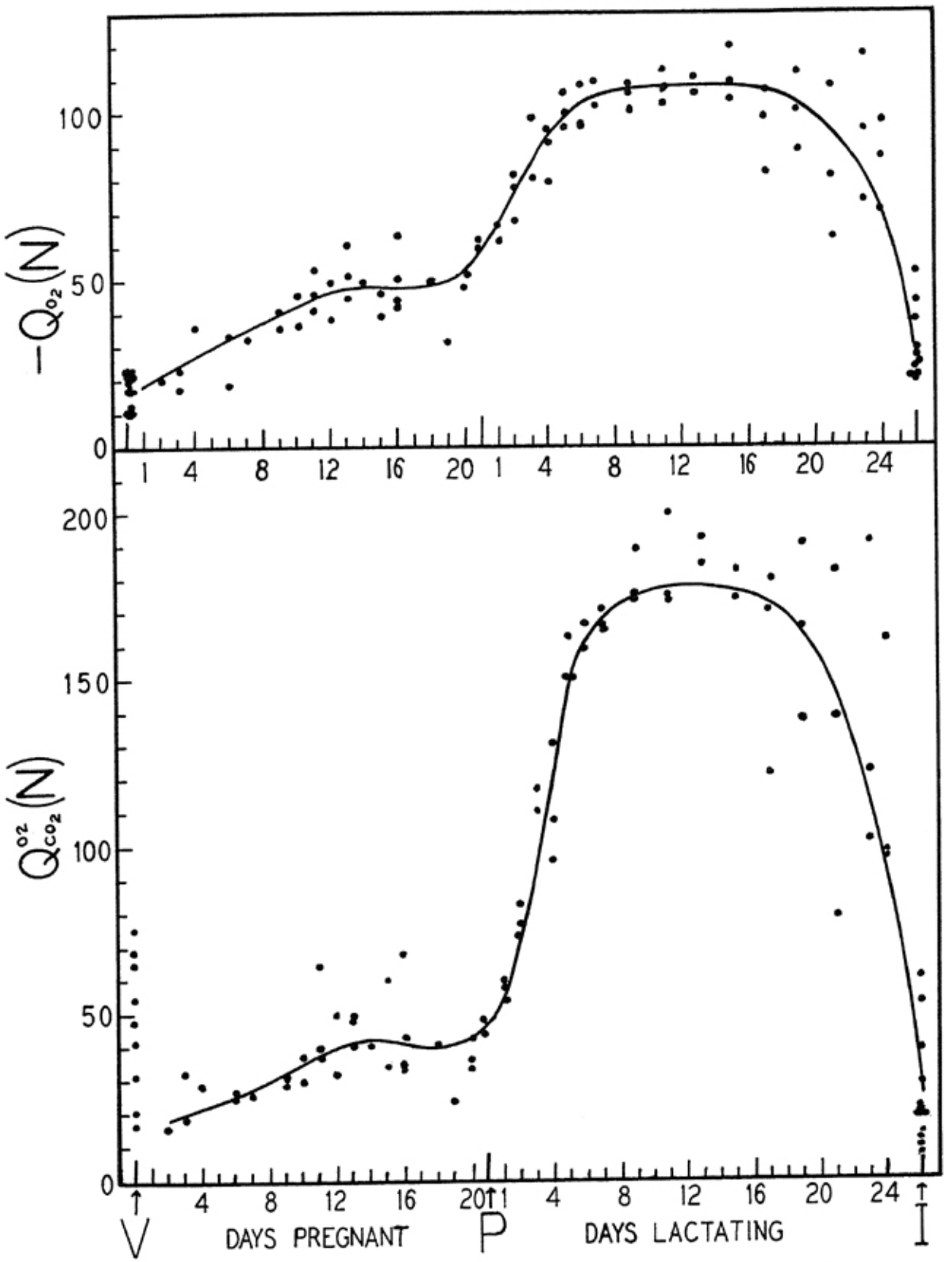

Fig. 4—Oxygen consumption and "metabolic" carbon dioxide production of rat mammary gland slices. V represents data on slices from virgin animals. P is time of parturition and I represents an involuted state.

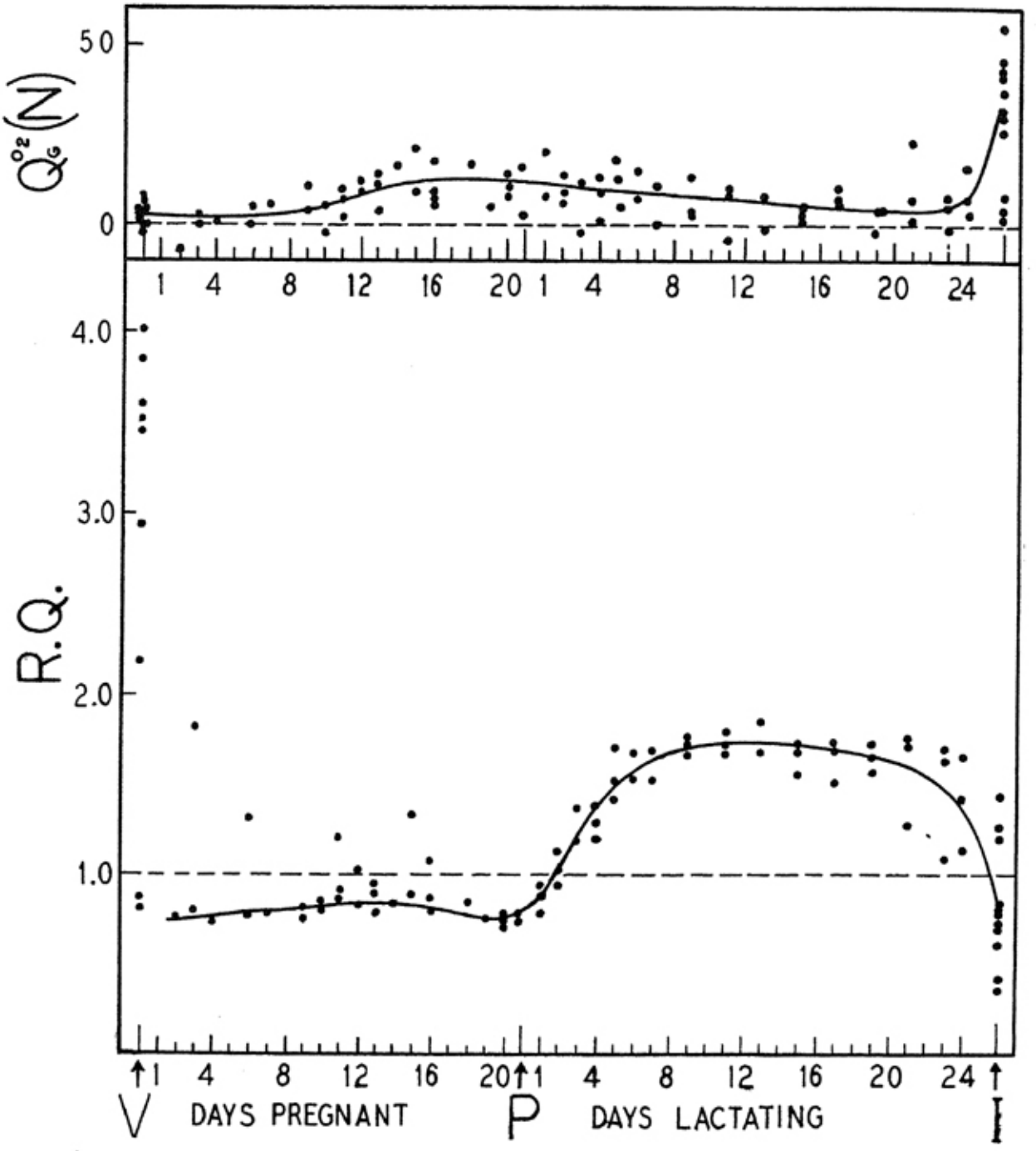

Fig. 5—Apparent aerobic acid production and respiratory quotient of rat mammary gland slices. Abscissa as in Fig. 4.

gin, non-estrous animals gave in several cases a remarkedly high R. Q. Here the slices represent a trivial amount of epithelial cells, i.e., measurements were essentially made on adipose tissue in spite of attempts to trim off excess fat and slice only in the region of a nipple. The adipose nature of the gland continues, to a gradually lessening extent, throughout the first part of pregnancy. The R. Q. of mammae from pregnant animals is characteristically in the normal range below one; however, high values were still encountered occasionally. In these cases, values are averages of multiple determinations; therefore, it is possible to observe an R. Q. above 1 in some mammae from pregnant rats (Fig. 5; Table 6). There may be a tendency for the R. Q. to rise slightly during mid-pregnancy. Just prior to parturition, values are close to 0.75. Within 2 days post-partum the quotient has reached 1 and is well on its way to a dramatic increase to about 1.7 at about the 8th day of lactation. As lactation proceeds values become less consistent but some remain high even at 24 days lactation.

Upon involution, the quotient in most cases fell to normal values but a few remained well above 1 and tissue from 2 animals had an R. Q. in the region of 0.4 (Fig. 5).

These data indicate that respiration increases about two-fold from the virgin state to mid- and late-pregnancy. From about the 16th to 20th day of pregnancy (a slight increase appears to occur just prior to parturition) to about the 7th day of lactation another 2½-fold increase was observed which tended to be variable during late lactation. Upon involution, oxygen consumption falls but not to the low values obtained from the virgin animals (Fig. 4; Table 6).

The "metabolic" CO_2 production from tissues of virgin animals was in general much greater than for early pregnant animals and had considerable variation over a wide range $\left(Q_{CO_2}^{O_2}\ (N) = 16 \text{ to } 75\right)$ (Fig. 4). Therefore, it is these values which are responsible for the extremely high R. Q. of mammary tissue from the virgin non-estrous rat. During pregnancy the carbon dioxide production in general follows the trend of respiration. However, following parturition it increases relatively more than respiration and results in high R. Q's within a few days post-partum. The trends in late lactation and involution conform to those of respiration but with a relatively greater drop in carbon dioxide production than in oxygen consumption upon involution.

Aerobic glycolysis tends to be very low throughout the conditions of this study with the exception of the involuted tissue where its carbon dioxide index tends to exceed the gas indices for respiration and "metabolic" carbon dioxide production. There may be a slight tendency for higher rates during the later part of pregnancy and the first part of lactation. When con-

TABLE 6 -- OXYGEN CONSUMPTION, "METABOLIC" CARBON DIOXIDE PRODUCTION, AEROBIC ACID PRODUCTION, R. Q., AND RATIO OF OXYGEN CONSUMPTION TO AEROBIC ACID PRODUCTION OF RAT MAMMARY GLAND SLICES. EACH LINE REPRESENTS DATA FROM ONE ANIMAL MEASURED IN AT LEAST DUPLICATE (AVERAGE) (EXCEPT FOR VIRGIN ANIMALS WHERE EACH LINE REPRESENTS ONE MEASUREMENT FROM ONE ANIMAL). DATA FOR FIGURES 6 AND 7.

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Physiological State of Animal	$-Q_{O_2}$ (N)	$Q^{O_2}_{CO_2}$ (N)	$Q^{O_2}_{G}$ (N)	R. Q. (Calculated)	$-Q_{O_2}:Q^{O_2}_{G}$ (Calculated)	Physiological State of Animal	$-Q_{O_2}$ (N)	$Q^{O_2}_{CO_2}$ (N)	$Q^{O_2}_{G}$ (N)	R. Q (Calculated)	$-Q_{O_2}:Q^{O_2}_{G}$ (Calculated)
Virgin	24	52	3	2.17	8.0	3 Days Lactating	81	111	-2	1.37	----
Virgin	24	21	2	0.88	12.0	4 Days Lactating	80	96	13	1.20	6.2
Virgin	22	76	0	3.45	----	4 Days Lactating	91	108	1	1.19	91.0
Virgin	21	17	7	0.81	3.0	4 Days Lactating	95	131	9	1.38	10.6
Virgin	18	65	4	3.61	4.5	5 Days Lactating	96	163	18	1.70	5.3
Virgin	19	69	-1	3.63	----	5 Days Lactating	107	151	5	1.41	21.4
Virgin	12	48	0	4.00	----	5 Days Lactating	100	151	12	1.51	8.3
Virgin	11	32	4	2.91	2.8	6 Days Lactating	96	160	7	1.67	13.7
Virgin	11	42	8	3.82	1.4	6 Days Lactating	109	167	15	1.53	7.3
2 Days Pregnant	21	16	-6	0.76	----	7 Days Lactating	101	171	11	1.69	9.2
3 Days Pregnant	18	33	3	1.83	6.0	7 Days Lactating	110	167	0	1.52	----
3 Days Pregnant	24	19	0	0.79	----	9 Days Lactating	99	174	13	1.76	7.6
4 Days Pregnant	37	29	1	0.78	37.0	9 Days Lactating	108	189	4	1.75	27.0
6 Days Pregnant	19	25	0	1.32	----	9 Days Lactating	106	176	3	1.66	35.4
6 Days Pregnant	34	27	5	0.79	6.8	11 Days Lactating	112	200	-4	1.79	----
7 Days Pregnant	33	26	6	0.79	5.5	11 Days Lactating	102	174	7	1.71	14.6
9 Days Pregnant	36	29	4	0.81	9.0	11 Days Lactating	106	175	10	1.65	10.6
9 Days Pregnant	41	31	11	0.76	3.7	13 Days Lactating	110	185	-1	1.68	----
10 Days Pregnant	46	38	6	0.83	7.7	13 Days Lactating	105	193	8	1.84	13.1
10 Days Pregnant	37	30	-2	0.81	----	15 Days Lactating	119	184	2	1.55	59.5
11 Days Pregnant	46	40	2	0.87	23.0	15 Days Lactating	103	175	5	1.70	20.6
11 Days Pregnant	54	65	7	1.20	7.7	15 Days Lactating	108	183	1	1.70	108
11 Days Pregnant	42	38	9	0.90	4.7	17 Days Lactating	107	180	10	1.68	10.7
12 Days Pregnant	39	32	9	0.82	4.3	17 Days Lactating	98	171	7	1.75	14.0
12 Days Pregnant	50	51	12	1.02	4.2	17 Days Lactating	81	122	6	1.51	13.5
13 Days Pregnant	52	49	4	0.94	13.0	19 Days Lactating	111	191	-2	1.72	----
13 Days Pregnant	45	41	13	0.91	3.5	19 Days Lactating	88	138	4	1.57	22.0
13 Days Pregnant	61	48	11	0.79	5.5	19 Days Lactating	100	166	4	1.66	25.0
14 Days Pregnant	49	41	16	0.84	3.1	21 Days Lactating	107	183	23	1.71	4.7
15 Days Pregnant	46	61	21	1.33	2.2	21 Days Lactating	62	79	7	1.27	8.9
15 Days Pregnant	39	35	9	0.90	4.3	21 Days Lactating	80	139	1	1.74	80
16 Days Pregnant	64	69	18	1.08	3.6	23 Days Lactating	117	191	-1	1.63	----
16 Days Pregnant	51	44	6	0.86	8.5	23 Days Lactating	73	123	8	1.69	9.1
16 Days Pregnant	43	34	7	0.79	6.1	23 Days Lactating	94	102	5	1.09	18.8
16 Days Pregnant	44	35	9	0.80	4.9	24 Days Lactating	70	99	7	1.41	10.0
18 Days Pregnant	49	41	17	0.84	2.9	24 Days Lactating	86	97	16	1.13	5.4
19 Days Pregnant	32	24	5	0.75	6.4	24 Days Lactating	97	162	3	1.67	32.3
20 Days Pregnant	47	34	14	0.72	3.4	Involuted	51	61	2	1.20	25.5
20 Days Pregnant	52	37	8	0.71	6.5	Involuted	19	8	37	0.42	0.5
20 Days Pregnant	56	43	10	0.77	5.6	Involuted	42	53	8	1.26	5.3
21 Days Pregnant	59	44	16	0.75	3.7	Involuted	26	21	30	0.81	0.9
21 Days Pregnant	62	48	3	0.77	20.7	Involuted	37	29	4	0.78	9.2
1 Days Lactating	62	58	20	0.94	3.1	Involuted	26	19	32	0.73	0.8
1 Days Lactating	67	60	8	0.90	8.4	Involuted	23	19	26	0.83	0.9
1 Days Lactating	69	54	11	0.78	6.3	Involuted	27	39	41	1.44	0.7
2 Days Lactating	82	83	14	1.01	5.9	Involuted	20	12	55	0.60	0.4
2 Days Lactating	68	77	6	1.13	11.3	Involuted	29	10	46	0.34	0.6
2 Days Lactating	78	73	9	0.94	8.7	Involuted	20	14	43	0.70	0.5
3 Days Lactating	99	118	12	1.19	8.4						

sidered with respect to oxygen consumption (Fig. 6) ($Q_{O_2} : Q_G^{O_2}$ ratio) this index is higher than reported by Folley and French (1949b) circles in Fig. 6; i.e., relative to oxygen consumption the aerobic glycolysis is lower than reported by these workers. Both sets of data show a tendency for relatively less acid production as lactation advances. Scattering of data when expressed as ratios in Fig. 6 is probably accounted for by the low absolute glycolytic rate. It is entirely possible that this index for glycolysis includes acid formation other than lactic acid.

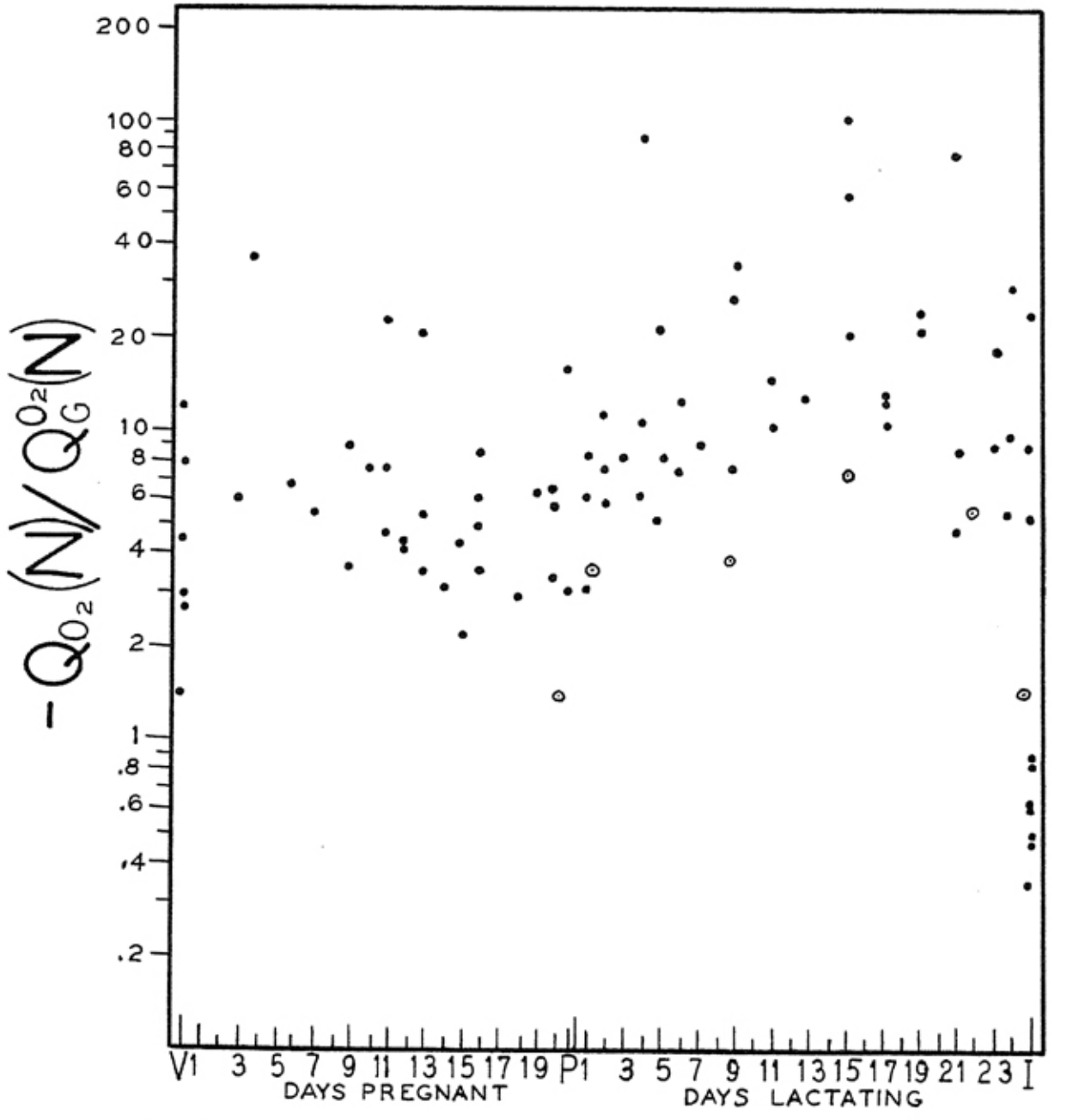

Fig. 6—Ratio of oxygen consumption to apparent aerobic acid production of rat mammary gland slices. Abscissa as in Fig. 4. Data given in Table 9. Circles represent data from Folley and French (1949b).

Adenosine Triphosphate Synthesis in Mammary Gland Homogenates

Among the many variations in the use of the yeast hexokinase glucose trapping system for measuring ATP synthesis, the one used by Hunter and Hixon (1949) was selected for the first experiments. Their procedure, slightly varied, follows: A basic reaction mixture was prepared in advance and stored in a deep-freeze unit for a period not exceeding two weeks. It contained each component in an amount such that 1.8 ml. represented enough material to give the indicated final concentration when diluted to 2.7 ml., the final reaction volume. Compounds and their final concentrations were; K_2HPO_4, 0.025 M; NaF, 0.04 M; AMP, 0.0015 M; DPN ("Sigma" 65 percent), 0.0002 M; cytochrome c, 0.000015 M; α-ketoglutaric acid, 0.01 M; and fructose, 0.03 M. The pH was adjusted to 7.42. Hexokinase ("Pabst" which is said to contain about 28,000 K. M. units per gm.) at a concentration of 10 mg. per ml. was prepared shortly before use. Enzyme preparations were obtained as follows: Quickly dissected tissue from rats killed by cervical dislocation was placed in the cold salt-buffer medium of Hunter (1949), containing phosphate, K^+, Na^+, and Mg^{++} at pH 7.6. Frequently the liver was perfused with cold medium prior to its excision. After cutting the tissue in rather small pieces, especially mammary gland tissue, homogenization in the same medium was achieved with a loose fitting Potter homogenizer (a close fitting one in the case of liver). The homogenate (ca. 5 gm. per 12 ml. medium) was forced through four layers of cheese cloth and centrifuged (10 min. at ca. 15,000 g). Floating fat and supernatant were discarded. The residue was resuspended and recovered two more times. Each time fat was removed by swabbing the tube wall with cotton, especially if mammary gland was being used. To the final residue ca. 5 ml. of medium was added. Brief homogenization was necessary for resuspension. To this suspension, additions of $MgSO_4$ were made such that when 0.5 ml. of the enzyme became diluted to 2.7 ml.—the final reaction volume—the concentration of Mg^{++} would be 0.006 M. This was necessary to keep from exceeding the solubility product of magnesium phosphate (Hunter and Hixon, 1949). All operations were carried out at temperatures as close to 0° C as possible. Centrifugations were at 5-7 ° C.

Each Warburg flask contained 1.8 ml. of basic reaction mixture, 0.5 ml. of Mg^{++}-enzyme preparation, 0.2 ml. of hexokinase, 0.2 ml. of water, KOH in center well, and 0.5 ml. of 50 percent trichlocoacetic acid in the side arm (later this was changed to 0.3 ml. of ca. 87 percent trichloroacetic acid). Temperature was 15.1° C during the mamometric measurements (some exceptions will be indicated). Flasks were placed in chipped ice while they were charged with all the reagents. Fifteen min. after they were placed in the Warburg apparatus, one pair of flasks was "killed" (trichloroacetic acid added to the main flask compartment) (controls) and returned to the

ice bath. Simultaneously, initial oxygen consumption readings were made on the remaining flasks and manometers. As was true for all experiments, the gas phase was air. At the end of the experimental period, remaining flasks were "killed" and treated in a manner similar to the controls (following recording of mamometric data).

Immediately upon "killing" a flask, it was mixed while in ice by repeatedly tipping the contents into the side arm. Without delay a portion of the mixture was diluted with acetate buffer (pH 4) and centrifuged and a supernatant was used for inorganic phosphorus analysis by procedure well adapted to low decomposition of unstable phosphate esters. (Lowry and Lopez, 1946). This phosphorus procedure was used in all experiments. Therefore, net phosphorus uptake (or release) during a time interval involving a measured oxygen consumption was attained. Control flasks were used for each set of experimental conditions.

The procedure was tested with rat liver. Oxygen consumption was linear over a 30-minute period, possibly longer. The P:O ratio (μM atoms oxygen consumed to net change—uptake in μM —of phosphorus) was found to approach 2, which is lower than values reported by Hunter and Hixon (1949) (Exp. 1 and 2; Table 7). Further experiments 3 and 4; Table 7), with the only modification in procedure being the addition of α-ketoglutarate as a separate component instead of being a member of the basic reaction mixture, show higher values approaching those reported in the literature.

TABLE 7 -- PHOSPHORUS UPTAKE BY WASHED LIVER RESIDUES
(Procedure of Hunter, et al., 1949; T=15.1°; α-ketoglutarate substrate)

Exp. No.	Exp. Time Interval	Modifier	-μM atoms oxygen	-net μM P	P:O
1a	40	none	8.66	12.9	1.49
1a	40	none	8.38	9.2	1.10
2a	30	none	2.99	5.8	1.94
2a	30	none	3.02	5.7	1.89
3a	50	none	4.46	12.0	2.69
3a	50	none	4.50	12.8	2.84
3b	50	triiodothyronine*	3.66	12.9	3.52
3b	50	triiodothyronine	3.52	11.1	3.15
3c	50	thyroxine*	4.13	13.8	3.35
4a	50	none	7.43	20.2	2.72
4a	50	none	7.62	20.2	2.65
4b	50	triiodothyronine*	6.58	19.7	3.00
4b	50	triiodothyronine	6.72	20.3	3.02
4c	50	thyroxine*	7.55	18.9	2.50
4c	50	thyroxine	7.73	16.4	2.12
5a	50	none	7.91	21.7	2.74
5a	50	none	8.42	21.0	2.50
5b	50	triiodothyronine*	7.50	21.8	2.91
5b	50	triiodothyronine	7.18	21.5	3.00
5c	50	thyroxine*	8.56	17.4	2.03
5c	50	thyroxine	8.70	19.0	2.18

* 1.1×10^{-5} final molar conc.

It was concluded that the procedure was suitable for study of a lactating mammary gland "washed residue." Such was not the case. An experiment (procedure identical to that used for Exp. 3) failed to give detectable oxygen consumption or change of inorganic phosphate level during a 40-min. incubation. Following this interval, the addition of sodium succinate gave slight oxidation upon further incubation. The final reaction mixture gave the appearance of curded milk, or, in fact, of protein precipitate (Exp. 4; Table 8). An additional experiment involving liver and mammary gland

TABLE 8 -- PHOSPHORUS UPTAKE BY LIVER (L) AND MAMMARY GLAND (M) FROM LACTATING RATS*

Exp. No.	days lactating	Modifier	Exp. time Interval	-μatoms oxygen	Net μM P	P:O
M4a	3	none	40	nil	nil	----
M4b	3	triiodothyronine	40	nil	nil	----
M4aa**	3	none	40	1.7	+0.9	----
M5a	6	none	40	0.25	+1.2	nil
M5a	6	none	40	0.31	+1.2	nil
L5a	6	none	40	5.62	-18.1	3.22
L5a	6	none	40	5.80	-17.9	3.19

* M4 series by procedure as used for Table 7; procedure for M5 series given in text.

** M4a flasks had sodium succinate added (0.01 M final conc.) after 40 min. and returned to bath for an additional 40 min.

tissue from the same animal showed slight oxygen consumption with a slight, but definite release of inorganic phosphate and confirmed previous results in other respects (Exp. M5 and L5; Table 8). These latter experiments were carried out with whole isotonic sucrose homogenates which gave a final mammary gland reaction mixture not showing protein flocculation. The resuspended liver residue was always light pink in color compared to a dull tan color of mammary gland suspensions, irrespective of suspending medium. Furthermore, homogenates of the latter tissue had a sharp disagreeable odor.

A continuation of these studies (same conditions as for Exp. 5) revealed that pyruvate, in absence of cosubstrate ("sparker"), was oxidized at a rate no greater than α-ketoglutarate (Exp. 6, Table 9; Experiment 20, Table 10). Confirmation of slow oxidation of succinate without simultaneous inorganic phosphorus uptake—a consistent observation in Experiments 6, 7, and 20—was obtained. Presence of nicotinamide and a liver coenzyme fraction ("Sigma") was without effect. Absence of hexokinase increased net release of inorganic phosphorus (Exp. M6bb; Table 9). Nicotinamide (0.01 M final concentration) was present in all subsequent homogenate experiments. Its use as an inhibitor of Co I destruction has been applied in other mammary gland studies since this tissue rapidly destroys the co-enzyme (Moore and Nelson, 1952; Terner, 1952).

TABLE 9 -- PHOSPHORUS UPTAKE BY MAMMARY GLAND TISSUE FROM LACTATING RATS (60 min. exp. time interval)*

Exp. No.	Substrate	-μ atoms oxygen	Net μM P	P:O
M6a	α-ketoglutarate	0.956	+0.5	---
M6a	α-ketoglutarate	0.937	+1.3	---
M6b	pyruvate	0.956	+1.3	---
M6bb**	pyruvate	0.813	+7.1	---
M6c	succinate	4.31	+0.5	---
M6c	succinate	4.37	+1.6	---
M7a	none	0.830	+1.5	---
M7a	none	0.812	+1.3	---
M7b	succinate	2.86	+0.6	---
M7b	succinate	2.58	+0.8	---
M7c***	succinate	2.73	+1.8°	---
M7c***	succinate	2.71	+1.6°	---

* Same conditions as for Table 8-5. Substrates at same molar level. Series 6 from 19 day lactating rat; series 7, 16 day lactating.
** No hexokinase.
*** Plus nicotinamide (0.01 M final conc.) and 2 mg of "Sigma" coenzyme fraction from liver per flask.
° Corrected for phosphate contributed by coenzyme addition.

TABLE 10 -- PHOSPHOROUS UPTAKE BY LIVER (L) AND MAMMARY GLAND (M) FROM 16 DAY LACTATING RAT; FURTHER CONFIRMATION OF THE MAMMARY GLAND'S INACTIVITY; 40% HOMOGENATE; OTHER CONDITIONS AS FOR TABLE 8-5 AND AS GIVEN IN TEXT

Exp. No.	Substrate	-μatoms oxygen	Net μM P	P:O
M20a	succinate	0.95	+ 0.7	--
M20a	succinate	1.02	+ 0.9	--
L20b	succinate	5.71	-12.4	2.17
L20b	succinate	5.68	-12.2	2.15
M20c	α-ketoglutarate	0.46	+ 3.4	--
M20c	α-ketoglutarate	0.66	+ 3.4	--
L20d	α-ketoglutarate	12.1	-32.5	2.68
L20d	α-ketoglutarate	12.4	-33.1	2.67

TABLE 11 -- PHOSPHORUS UPTAKE BY MAMMARY GLAND (M), LIVER (L), AND MAMMARY GLAND PLUS LIVER TISSUE FROM LACTATING RAT;* SUCCINATE SUBSTRATE; EXP. TIME INTERVAL WAS 50 MIN.**

Exp. No.	Enzyme	-μatoms oxygen	Net μM P	P:O
8a*	0.4 ml M	3.46	+ 0.3	--
8a	0.4 ml M	3.31	+ 0.7	--
8b	0.4 ml L	11.0	-16.4	1.49
8b	0.4 ml L	10.5	-15.7	1.50
8c	0.2 ml M + 0.2 ml L	10.8	-13.6	1.26
8c	0.2 ml M + 0.2 ml L	10.3	-12.9	1.25
9a*	0.4 ml M	2.93	+ 1.6	--
9a	0.4 ml M	2.86	+ 2.0	--
9b	0.4 ml L	12.4	-22.0	1.77
9b	0.4 ml L	12.3	-21.0	1.71
9c	0.2 ml M + 0.2 ml L	11.9	-16.4	1.38
9c	0.2 ml M + 0.2 ml L	11.7	-17.6	1.50

* Series 8 from 14 day lactating rat; series 9 from 11 day lactating animal.
** Exp. conditions as for Table 8-5.

By mixing a highly active liver preparation (conditions as for Exp. 5) with an inactive mammary gland resuspended residue, it was learned that the mixture's activity was greater (Exp. 8c and 9c) than would be expected from measurements of both preparations alone (Exp. 8a, 8b, 9a, and 9b; Table 11). Since measurements of the uncombined preparations were made upon a relative enzyme concentration of four and of two (each) when combined, it was necessary to know the extent of dependence upon enzyme "concentration." By study of oxidative phosphorylation upon serial dilution of a whole 30 percent (wet wt.) isotonic sucrose homogenate, it was clearly shown that phosphorus uptake became zero at an enzyme level which still gave an oxygen consumption that would, if the P:O ratio remained constant, permit easy detection of inorganic phosphate uptake; i.e., P:O ratio decreased as the enzyme concentration decreased (Fig. 7; Table 12).

TABLE 12 -- OXIDATIVE PHOSPHORYLATION OF WHOLE LIVER HOMOGENATE; AVERAGE OF DUPLICATE MEASUREMENTS; EXPERIMENTAL CONDITIONS AS FOR EXP. 5, TABLE 11; DATA FOR FIG. 7.

Homogenate Concentration (% wet wt.)	0_2 consumed μl	P uptake μM	P:O
30	124	18.2	1.64
15	81.7	62.0	1.52
7.5	62.0	6.3	1.14
3.3	27.0	0	0

TABLE 13 -- OXIDATION OF KREBS CYCLE INTERMEDIATES BY MAMMARY GLAND FROM LACTATING RATS; 30% ISOTONIC SUCROSE HOMOGENATE; T=38°; OTHER CONDITIONS GIVEN IN TEXT.

Exp. No.	Substrate (final conc.) 0.1 M	0.01 M	-μl 02/hr/100 mg wet wt.
11*	none	none	37.5
11	pyruvate	none	39.0
11	pyruvate	fumarate	38.0
11	pyruvate	succinate	39.4
11	α-ketoglutarate	none	31.2
11	α-ketoglutarate	fumarate	31.6
11	α-ketoglutarate	succinate	40.8
11	citrate	none	29.6
11	citrate	fumarate	33.6
11	succinate	none	168.0
11	fumarate	none	35.1
11	fumarate	pyruvate	43.0
12**	none	none	33.8
12	pyruvate	none	39.7
12	pyruvate	0.05M $NaHCO_3$	65.0
12	pyruvate	0.10M $NaHCO_3$	64.1
12	pyruvate	0.20M $NaHCO_3$	73.4
12	succinate	none	155.0
12	pyruvate	acetate	38.2
12	citrate	none	30.4
12	citrate	acetate	37.6
12	citrate	0.1M $NaHCO_3$	50.1

* 9 day lactating rat
** 15 day lactating rat

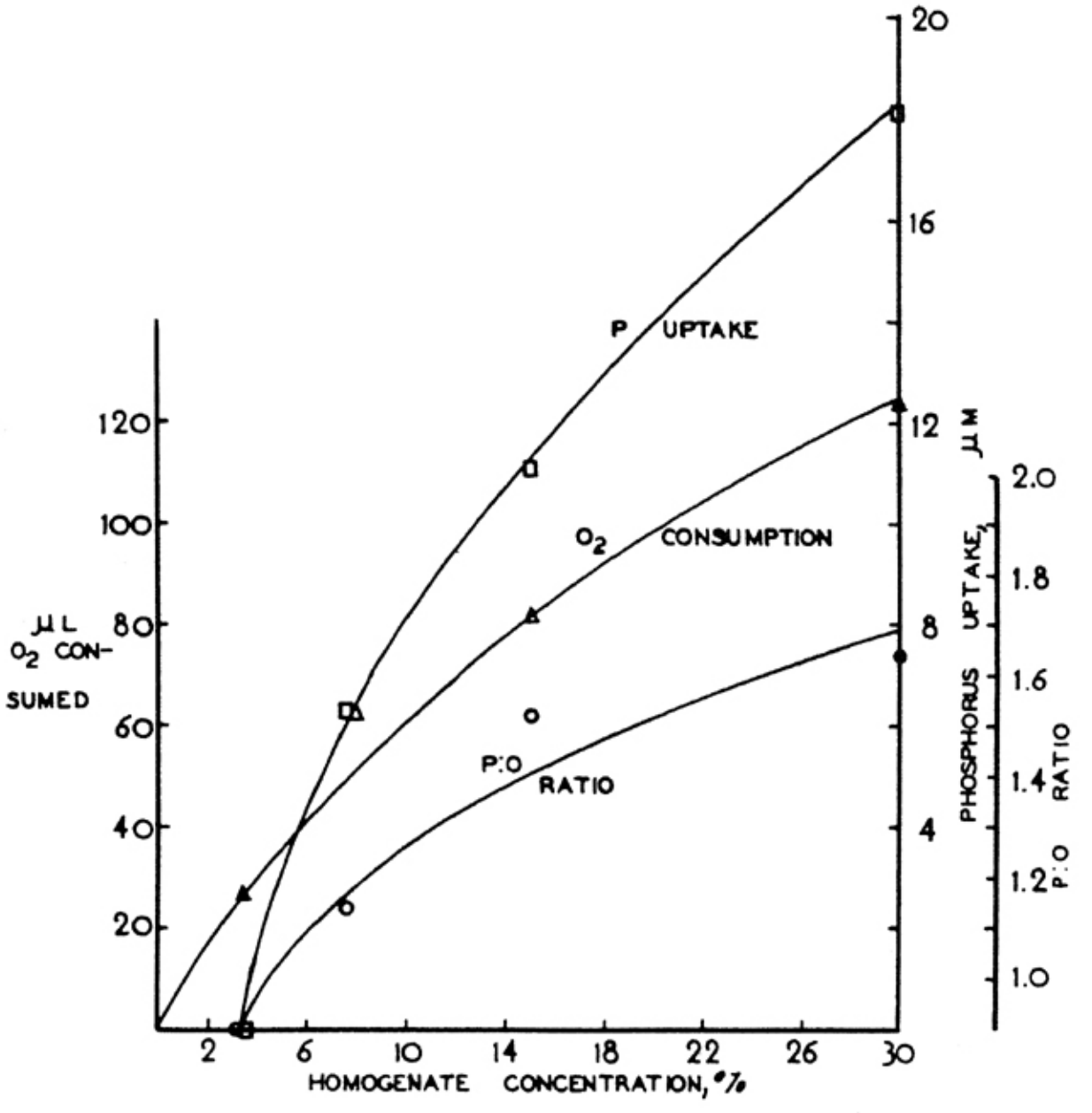

Fig. 7—Dependence of oxygen consumption, phosphorus uptake, and P:O ratio of rat liver resuspended residue upon enzyme "concentration." Data from Table 15.

Since oxidation of some Krebs cycle intermediates by cell free mammary gland preparations has been reported (Moore and Nelson, 1952), the oxidation of pyruvate, α-ketoglutarate, citrate, fumarate, and succinate, with and without cosubstrates ("sparkers") were studied (T=38°C; other conditions as Exp. 5 plus nicotinamide). Among these substrates, again only succinate was oxidized to any measurable extent when in absence of "sparker." Even oxidation of this substrate was slow. Some slow oxidation of pyruvate above endogenous rates (to a less extent with citrate) in presence of bicarbonate was observed (Exp. 11 and 12; Table 13).

Further attempts to obtain better oxidation of succinate, α-ketoglutarate, and OAA were made by using the following procedure: Rat mammary glands, lactating 15 days, were homogenized as previously described in a medium containing KCl and Na_2HPO_4 (0.129 M and 0.013 M, respectively) at pH 7.8. The homogenate was sedimented three times and resuspended in a volume of medium equal to the wet weight of the initial tissue. To 1 ml. of this enzyme preparation was added, in a Warburg flask, 1 ml. of substrate containing 30 μ M of neutralized substrate; 0.3 ml. of 0.1 M K_2HPO_4 at pH 7.4; 0.3 ml. of 0.01 disodium ATP; 0.1 ml. of 0.05 M $MgSO_4$; and 0.3 ml. of water. Except for using rat rather than guinea pig tissue, this represents only slight deviation from Moore and Nelson's (1952) procedure. Oxygen consumption was measured at 38° C which was found to be less than 2 μ l during a 30 minute incubation. Such a preparation carried little oxidative activity.

Attempts to observe oxidative phosphorylation by 40 percent whole isotonic sucrose homogenates (conditions as for Exp. 5) of lactating mammary glands of other species (goat, rabbit, cat, and guinea pig) also met with failure (Exp. 14, 15, 16, 17, and 18; Table 14). However, such a preparation from the guinea pig was able to oxidize HDP with a detectable inorganic phosphorus uptake. This has been reported recently for guinea pig mammary gland tissue (lactating) (Terner, 1954). A pair of experiments

TABLE 14 -- PHOSPHORUS UPTAKE BY LACTATING MAMMARY GLAND PREPARATIONS FROM VARIOUS SPECIES; 40% HOMOGENATES; OTHER CONDITIONS AS FOR TABLE 8-5.

Exp. No.	Species	Substrate	-μ atoms oxygen	Net μ M P	
14a+	goat*	none	0.47	+1.5	
14b	goat	α-ketoglutarate	1.01	+1.6	
14c	goat	succinate	1.44	+1.8	
15**	rabbit **				
16***	cat***				
17a	cat++	none	0.33	+1.9	
17b	cat	α-ketoglutarate	0.52	+2.3	
17c	cat	succinate	2.48	+1.9	
18a	guinea pig	succinate	nil	+2.8	
18b	guinea pig	α-ketoglutarate	nil	+2.0	
18c	guinea pig	none	nil	+2.3	
19ax	guinea pig	hexosediphosphate	1.89	-1.4	
19b	guinea pig	hexosediphosphate	1.93	-5.6	(no hexokinase)
19c	guinea pig	hexosediphosphate	2.07	-2.6	(no F^-)

* ca. 3 mo. post-partum

\+ average of three measurements; except Exp. 18 and 19

** Same as Exp. 14; ca. 20 day post-partum; no oxidation of either substrate observed during a 20 min. interval.

*** Same as Exp. 14; ca. 3-4 weeks post-partum; no oxidation of either substrate observed in a 30 min. interval; gland appeared to be poorly developed or involuted.

++ ca. 2.5 weeks post-partum; obvious good milk secretion

x Same tissue preparation as used in Exp. 18 with a delay of ca. 1.5 hr.

TABLE 15 -- PHOSPHORUS UPTAKE BY LACTATING RAT MAMMARY GLAND HOMOGENATES (40%) IN PRESENCE OF HEXOSEDIPHOSPHATE; CONDITIONS AS FOR EXP. 19, TABLE 14.

Exp. No.	Omission from complete system	-μ atoms oxygen	Net μM P	P:O
21a*	none	3.06	-5.3	1.73
21a	none	3.08	-5.4	1.75
21b	hexokinase	2.02	+0.1	--
21b	hexokinase	1.91	+0.1	--
21c	F^-	0.89	+0.2	--
21c	F^-	0.92	+0.5	--
21d	F^- and hexokinase	0.39	-1.3	--
21d	F^- and hexokinase	0.48	-0.8	--
22a**	none	4.51	-9.9	2.20
22a	none	4.16	-8.0	1.93
22b	hexokinase	2.84	-2.0	0.70
22b	hexokinase	2.75	-1.4	0.59
22c	F^-	1.79	+0.8	--
22c	F^-	2.05	+0.4	--
22d	F^- and hexokinase	1.59	+5.7	--
22d	F^- and hexokinase	1.46	+7.9	--

* 11 days post-partum
** 7 days post-partum

using HDP substrate, sucrose homogenate, and other conditions as for Exp. 5 gave P:O ratios above 1.7 in the complete system. Phosphorus uptake was highly dependent upon presence of hexokinase and fluoride ion. Highest rates of oxygen consumption were also observed in presence of the complete system (Exp. 21 and 22; Table 15). Under similar conditions, but at 30° C, another preparation was found to be unstable as reflected by strongly depressed rates of oxygen consumption, even after 15 minutes of incubation (Table 16).

TABLE 16 -- INSTABILITY OF PHOSPHORUS UPTAKE BY MAMMARY GLAND HOMOGENATE (33.3% FROM 16 DAY LACTATING RAT) AT 30°; HEXOSEDIPHOSPHATE SUBSTRATE; OTHER CONDITIONS AS DESCRIBED FOR TABLE 15.

Exp. time interval (min.)	-μ atoms* oxygen	Net μM * P
15	1.98	-0.3
30	2.80	-3.5
45	3.30	-3.0
60	3.24	-1.8
75	3.35	-2.3

* Average of duplicate measurements except for 75 min. interval

At this point procedural details were modified for parsimonious reasons. They were as follows: The basic reaction mixture (pH 7.5) contained a series of reagents in which 1.3 ml. represented amounts of each member when diluted to 2.7 ml. as follows: Disodium ATP, 0.001 M; glucose, 0.026 M; cytochrome c, 0.000015 M; KCl, 0.025 M; K_2HPO_4, 0.02 M; and

nicotinamide, 0.04 M. This mixture was stored as previously indicated. The final reaction mixture contained (in order of addition to reaction flask) 1.3 ml. of above basic reaction mixture; 0.3 ml. of HDP, 0.002 M; 0.2 ml. containing 2 mg. hexokinase ("Pabst"); 0.1 ml. Co I, 0.0002 M; 0.4 ml. enzyme (or more with proper volume and concentration compensations); 0.1 ml. NaF, 0.013 M; 0.2 ml. $MgSO_4$, 0.0067 M; 0.1 ml. water or experimental modifier (or its solvent); 0.3 ml. trichloroacetic acid (ca. 87%) in side arm; and 0.2 ml. KOH (ca. 15%) in center well. Molar concentrations were again those of the final mixture. Homogenates were prepared in isotonic sucrose (0.25 M) as described previously and used without sedimentation of a residue. Incubation was carried out at 15.1° C.

This complete system was not able to significantly take up inorganic phosphate when the enzyme source was rabbit (lactating 16 days) mammary gland. However, for some unknown reason, omission of the fluoride ion resulted in phosphate uptake without much alteration of the low oxygen consumption (Exp. M23A; Table 17). The same enzyme preparation

TABLE 17 -- PHOSPHORUS UPTAKE BY A LACTATING (16 day) RABBIT (EXP. 23) MAMMARY GLAND PREPARATION AND LACTATING (9 day) RAT LIVER AND MAMMARY GLAND (EXP. 24); MODIFIED CONDITIONS GIVEN IN TEXT.

Exp. No.	Omission from complete system	-μ atoms oxygen	Net μM P	P:O
M23Aa*	none	1.67	-0.4	--
M23Ab	hexosediphosphate	1.11	+0.1	--
M23Ac	hexokinase	0.88	+3.2	--
M23Ad	F^-	1.39	-3.8	2.83
M23Ae	F^- and hexokinase	1.07	+0.1	--
M23Ba*	none	3.87	-5.7	1.47
M23Bb	hexosediphosphate	1.25	-0.5	--
M23Bc	hexosediphosphate (plus pyruvate)	1.37	-1.5	--
M23Bd	complete (plus pyruvate)	2.36	-3.6	1.53
M23Be	complete (plus α-ketoglutarate)	2.91	-2.7	--
L24a*	none	6.38	-9.9	1.55
M24a	none	2.84	-4.6	1.62
M24b	none (plus DNP°)	2.68	-4.5	1.68

* average of duplicate determinations except Exp. M23Bc and M24a.
° 3×10^{-5} final molar conc.

was held in ice for somewhat less than two hours, then studied further (Exp. M23B; Table 17) under similar experimental conditions. This time, the complete system was able to take up significant amounts of inorganic phosphate which was accompanied by a higher rate of oxygen consumption. Consistent phosphate uptake was observed by lactating rat mammary gland preparations (Exp. M24, Table 17; Exp. 24 and 27, Table 18). DNP (3×10^{-5} or 10^{-4} M) had little effect on the P:O ratio (Exp. M24b and 27 c; Tables 17 and 18, respectively). IAA (iodoacetic acid) obliterated phosphate uptake and reduced oxygen consumption to a level two-thirds of the en-

TABLE 18 -- PHOSPHORUS UPTAKE BY RAT MAMMARY GLAND HOMOGENATE (33.3%); SAME CONDITIONS AS FOR TABLE 17.

Exp. No.	Substrate	Modifier	-μ atoms oxygen	Net μM P	P:O
24a	none	none	5.52	- 2.9	0.53
24a	none	none	5.42	- 2.5	0.46
24b	HDP	none	8.36	- 8.9	1.06
24b	HDP	none	8.31	- 9.4	1.13
24c	HDP	DPN*	8.43	-10.3	1.22
24c	HDP	DPN*	8.39	-10.5	1.25
24c	HDP	DPN*	8.13	-10.2	1.26
24d	DL-glyceraldehyde°	none	3.55	- 1.0	0.29
24d	DL-glyceraldehyde°	none	3.93	- 1.2	0.31
27a	none	none	4.09	- 2.7	0.66
27a	none	none	3.93	- 3.5	0.89
27b	HDP	none	7.91	- 8.2	1.04
27b	HDP	none	7.60	- 8.4	1.10
27c	HDP	DNP**	7.48	- 8.6	1.15
27c	HDP	DNP**	7.43	- 8.2	1.10
27d	HDP	IAA^x	2.30	+ 1.3	---
27d	HDP	IAA^x	2.36	+ 0.5	---
27e	glycerol°	none	4.29	- 3.1	0.72
27e	glycerol°	none	4.36	- 2.9	0.67

* 1×10^{-4} final molar conc. x 0.5×10^{-3} final molar conc.
° 0.02 final molar conc. ** 3×10^{-5} final molar conc.

dogenous rate. In all these cases the presence of only HDP increased oxygen consumption by a factor of about two above the endogenous rate. Presence of glycerol produced results nearly identical to those reactions being carried out in absence of substrate (HDP) (Exp. 27e; Table 18).

Since consistent failure was met in obtaining reasonable oxidation of Krebs cycle intermediates by cell free, lactating rat mammary gland tissue, a series of experiments were made on such preparations which were carried out in presence of ethylenediaminetetraacetic acid (EDTA). Sarcosomes isolated in presence of this chealating agent have been shown to be much more stable and active than those isolated in its absence (Slater and Holton, 1954).

Enzyme preparations for the following experiments (Exp. 41 through 46; Tables 19 through 21) were "washed residues" from homogenates made in isotonic sucrose containing 0.01 M EDTA adjusted to pH 7.5 with KOH. Residues were sedimented three times with removal of supernatant following each centrifugation and finally resuspended in a volume of medium (sucrose plus EDTA) equal to ca. 1½ times the original wet weight of tissue.

Microscopic examination of this suspension showed considerable similarity among different preparations. Wet smears stained with pyronine B and methyl green showed vast numbers of unclumped small pyronine B stained particles (mitochondria ?). Shreds of connective tissue (various sizes) stained likewise. They were observed fairly frequently. No methyl

TABLE 19 -- OXIDATION OF KREBS CYCLE INTERMEDIATES BY "WASHED RESIDUES" OF LACTATING RAT MAMMARY GLANDS PREPARED IN PRESENCE AND ABSENCE OF ETHYLENEDIAMINETETRAACETIC ACID (EDTA); EXPERIMENTAL CONDITIONS GIVEN IN TEXT.

Exp. No.	Substrate	$-Q_{O_2}$ (N) (average of duplicates) EDTA absent	EDTA present
41	none	nil	2.3
41	α-ketoglutarate	4.7	8.7
42	none	nil	nil
42	α-ketoglutarate	nil	10.0
42	succinate	63.4	26.7
42	OAA	nil	9.4
43	none	3.9	3.3
43	α-ketoglutarate	10.0	42.4
43	succinate	25.4	63.9

TABLE 20 -- OXIDATIVE PHOSPHORYLATION BY "WASHED RESIDUES" FROM HOMOGENATES OF LACTATING RAT MAMMARY GLANDS IN PRESENCE OF EDTA; AVERAGE OF DUPLICATES.

Exp. No.	Substrate	-μ atoms oxygen	Net μM P	P:O	$-Q_{O_2}$ (N)
44a	none	1.48	+ 0.1	----	10.8
44a	succinate	9.46	-11.0	1.16	69.6
44a	α-ketoglutarate	5.58	-11.7	2.10	40.0
44a*	α-ketoglutarate	4.64	- 2.6	0.50	34.1
44b	none	1.24	- 1.4	----	9.1
44b	succinate	9.11	-12.2	1.34	66.9
44b	α-ketoglutarate	5.58	-11.0	1.97	40.0
44b*	α-ketoglutarate	4.54	- 2.1	0.46	33.3

* DNP at 10^{-4} final molar concentration also added

TABLE 21 -- EFFECT OF THYROID HORMONES ON OXYGEN CONSUMPTION OF "WASHED RESIDUE" FROM HOMOGENATES OF LACTATING RAT MAMMARY GLANDS; α-KETOGLUTARATE SUBSTRATE.

Exp. No.	No. of Determinations	Modifier	$-Q_{O_2}$ (N) (average with average deviation)
45a	4	none	53.0 $\pm$ 2.4
45b	2	L-thyroxine*	48.4 $\pm$ 0.8
45c	2	L-triiodothyronine*	55.8 1.4
45d	2	prolactin**	52.9 4.2
46a	2	none	44.4 0.7
46b	3	L-thyroxine*	45.4 3.2
46c	2	L-triiodothyronine*	47.0 0.6

* 10^{-5} final molar concentration
** ca. 0.5 I. U./ml, final concentration

green stained bodies which could be called free nuclei, single cells, or groups of a few intact cells were ever seen. However, groups of intact cells containing perhaps two dozen nuclei were seen with moderate frequency. This is in contrast to liver where incomplete homogenization results in cells and clumps of cells in all stages of disintegration. Another feature was the vast number of extremely small fat droplets. Some approached the size of nuclei. Therefore, centrifugation fails to remove a good measure of the large quantities of fat in these homogenates. Of course, many particles of glass showing conchoidal fracture are present in these preparations.

Although the first experiments involving oxidation of some Krebs cycle intermediates gave low rates of oxygen consumption, the hint that the presence of EDTA increased these rates was clear (Exp. 41 and 42; Table 19). The utility of EDTA became definite with Expperiment 43 (Table 19) where its presence resulted in ca. 4 fold increase of α-ketoglutarate and ca. 2½ fold increase in the case of succinate oxidation. Experimental conditions were as follows: The final reaction mixture (2.7 ml.) contained 1.3 ml. of the basic reaction mixture last described for Experiment M23A (Table 17); 0.3 ml. substrate at a concentration such that the final mixture was 0.02 M; 0.1 ml. Co I (0.0002 M final concentration); 0.4 ml. enzyme; 0.6 ml. water; and other usual conditions for aerobic manometry. Incubation was at 38° C. Each flask contained ca. 3 mg. of enzyme nitrogen. It was determined colorimetrically on an aliquot of the final enzyme suspension as described for mammary gland slices. Experiment 41 involved tissue from a rat lactating 16 days; Experiment 42, 11 days; and Experiment 43, 13 days.

By the procedure for Experiment M23A the ability of these stablized residues to carry out oxidative phosphorylation in presence of succinate and α-ketoglutarate was studied (Exp. 44a and 44b; Table 20). Both experiments showed moderately active inorganic phosphate uptake which was strongly depressed but not obliterated by DNP at 10^{-4} M level. The fact that both experiments were made on tissue from rats lactating 10 days probably contributed to the good agreement.

Two more experiments intended to further confirm observations of oxidative phosphorylation met with partial failure. Phosphorus samples were lost for Experiment 45 (Table 21) (rat lactating 8 days) and no uptake, in fact, a slight release was observed in Experiment 46 (Table 21) (rat lactating 6 days). However, oxygen rate coefficients were in agreement with Experiments 44a, 44b, and 43. In Experiments 42 and 46 it was not possible to carry out centrifugations in the "cold-room" at temperatures below 14° C.

The physical appearances of the resuspended residues were obviously different from those prepared in absence of EDTA. Although the latter were usually dull tan to brown (sometimes quite white) in color, the material obtained from EDTA containing isotonic sucrose was light pink and reminded one of liver preparations used in the early part of this work.

In vitro Influence of Triiodothyronine on Some Metabolic Processes

Since thyroid hormones (at least thyroxine) are galactopoietic and 3, 5, 3'-L-triiodothyronine has recently been characterized as a thyroid hormone, its *in vitro* influence on some metabolic processes was studied. The main question is whether this compound, *in vitro,* is able to stimulate respiratory processes in surviving tissue during short time (one-hour) incubation, a phenomenon not observed with L-thyroxine in spite of its *in vivo* ability to achieve such respiratory stimulation.

Tissues (rat) were sliced as previously described for mammary gland tissue and incubated one hour at 38° C in Krebs-Ringer-phosphate medium (pH 7.34) containing 0.2 percent glucose or molar equivalent of other substrates. Oxygen consumption was measured in presence of air and other usual standard mamometric condition (Umbreit, *et al.,* 1949). L-Thyroxine or L-triiodothyronine (dissolved in 0.01 N NaOH) was added in 0.1 ml. to 2.9 ml. medium at concentrations that gave the indicated final concentrations. Control flasks contained an equivalent amount of NaOH. Tissue was removed from each flask following the experiment and dried at 105° C for 2½ hours for measurement of dry weights, the reference base for all these studies except the yeast experiment (Table 24).

Effect of triiodothyronine (10^{-8} to 10^{-4} M final concentration) was studied on young mature rat liver slices (10 animals, glucose substrate). With the observation that a respiratory coefficient of 56.2 (average) at 10^{-5} M triiodothyronine compared to 50.5 for tissue from the same animals, it appeared that this thyroid hormone did indeed slightly augment *(in vitro)* respiration (Table 22). It was thought that if the tissue came from hypothyroid animals, this effect, if it was real, might be more pronounced. Therefore, similar studies were repeated on animals which had 0.1 percent thiouracil in their feed for 21 days prior to experimentation. The thyroid weight was indicative of their extensive hypothyroidism. Also the average control (tissue from hypothyroid animal in absence of added hormone) had a low respiratory coefficient (36.2), compared to the value (50.5) for analogous normal animals. However, the *in vitro* presence of triiodothyronine was without effect on such tissue's respiration (Table 22).

Similar studies were made on normal kidney slices (experimental conditions unchanged) involving glucose, pyruvate (alone), and succinate substrates. At 10^{-4} M, triiodothyronine was without effect on oxidation of any of these metabolites (Table 23). This was also true for baker's yeast ("Red Star") which was permitted to actively ferment in glucose containing Krebs-Ringer-phosphate prior to resuspension in this medium containing glucose or pyruvate (alone) and measuring respiration in absence and presence

TABLE 22 -- IN VITRO INFLUENCE OF TRIIODOTHYRONINE ON RESPIRATION OF SLICES; NORMAL (C SERIES) AND HYPOTHYROID (E SERIES) RAT LIVER; MALES; GLUCOSE SUBSTRATE; KREBS-RINGER- PHOSPHATE MEDIUM

Exp. No.	gm body wt.	thyroid wt.*	$-Q_{O_2}$/hr/10 mg dry wt.++ Final molar conc. of triiodothyronine 0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
E1	128	22.0	38.4 ± 0.4	32.9 ± 1.2		39.1 ± 4.1	32.6 ± 0.5	31.7 ± 1.7
E2	146	26.3	25.6 ± 1.1	28.1 ± 2.0		37.2 ± 3.0	46.6 ± 5.6	35.1 ± 1.6
E3	132	30.1	26.4 1.0	32.8 2.2			29.2 1.1	30.4°
E4	146	20.2	26.4 2.7	28.5 1.2		30.6 0.6	24.0 0.1	28.0°
E5	150	18.5	37.3 1.3	30.7 0.7		36.9 1.6	35.6 0.8	31.7 1.7
E6	212	24.4	41.2 3.8				33.4 4.5	
E7	214	21.5	41.1 3.6				42.7 3.4	
E8	223	24.1	46.4 0.5				49.0 0.8	
E9	205	24.7	44.0 5.4				49.2 4.2	
Average	173	23.5	36.2	30.6		35.9	38.0	31.4
C1	149	10.7	36.7 ± 4.7	40.2 ± 5.1		33.8 ± 0.4	34.3 ± 2.5	32.0 ± 0.8
C2	175	6.0	44.6 ± 3.4	46.8 ± 3.0		53.1 ± 1.6	51.8 ± 4.2	52.2 ± 1.2
C3	176	9.4	53.6 0.4	53.0 0.4		58.9 5.8	67.0 1.0	63.6 0.2
C4	216	8.5	52.8 2.7	50.9 1.3		55.4 1.6	56.5 3.3	54.8 3.9
C5	240	8.5	56.4 0.5	56.5 2.7		61.5 2.3	64.3 1.6	65.5 1.6
C6	171	5.6	48.0 6.6		58.6 ± 1.5	52.5 1.4	56.8 1.9	60.7 1.4
C7	225	6.9	57.6 4.0		57.3 ± 0.5	57.8 0.2	65.3 1.5	57.8 1.8
C8	202	9.0	42.8 3.5		39.8 1.1	58.0 5.5	52.3 4.6	44.5 4.7
C9	207	6.7	56.7 0.9		46.6 0.9	58.2 4.4	52.6 1.2	57.5 2.4
C10	238	8.0	55.6 2.2		55.8 2.6	57.7 5.5	61.2 3.5	63.9 4.6
Average	200	7.9	50.5	49.5	51.6	54.7	56.2	55.3

* mg/100 gm body wt.
++ duplicate measurement with average deviation
° single measurement

TABLE 23 -- IN VITRO INFLUENCE OF TRIIODOTHYRONINE ON RESPIRATION OF NORMAL MALE RAT KIDNEY SLICES; KREBS-RINGER-PHOSPHATE MEDIUM

Animal No.	Gm body wt.	Substrate	$-Q_{O_2}$/hr/mg dry wt.** Final Molar conc. of triiodothyronine 0	10^{-6}	10^{-4}
83	163	glucose	18.0 ± 0.0	18.3 ± 0.5	14.3*
84	223	glucose	18.4 0.6	17.8 0.5	16.6 ± 0.4
85	193	glucose	19.4 0.0	15.2 0.3	16.8 0.2
86	124	glucose	21.2 0.1	22.6 1.4	18.3 0.6
88	163	glucose	18.2 1.2	20.1 1.5	15.8 0.2
Average	173	glucose	19.0	18.8	16.3
89	297	glucose	14.7 ± 0.5		14.0 ± 1.1
89		pyruvate	22.2 0.1		20.8 0.9
89		succinate	34.0 0.8		26.1 1.5
90	331	glucose	17.0 0.3		16.0 0.9
90		pyruvate	19.7 1.4		19.0 0.2
90		succinate	26.2 2.2		34.3 1.3
91	315	glucose	13.3 0.3		16.9 0.6
91		pyruvate	24.9 0.7		22.2 1.0
91		succinate	31.3 1.1		35.7 0.6
92	368	glucose	16.0 0.2		17.2 0.8
92		pyruvate	23.5 0.7		23.3 0.1
92		succinate	31.0 2.5		26.3 0.4
Average	328	glucose	15.2		16.0
		pyruvate	22.6		21.3
		succinate	30.5		30.6

** duplicate measurement with average deviation
* single determination

TABLE 24 -- IN VITRO INFLUENCE OF THYROXINE AND TRIIODOTHYRONINE ON RESPIRATION OF BAKER'S YEAST; KREBS-RINGER PHOSPHATE MEDIUM

Modifier	Substrate	$-\mu l\ O_2$/hr* Final molar conc. of modifier 0	10^{-5}	10^{-4}
triiodothyronine	glucose	306 ± 0.5	292 ± 4.0	294 ± 2.0
thyroxine	glucose	306 0.5	296 1.5	298 2.0
triiodothyronine	pyruvate (only)	99.0 0.1	98.9 0.2	99.2 1.8
thyroxine	pyruvate (only)	99.0 0.1	98.4 1.2	100.6 0.4

* duplicate or triplicate measurement with average deviation

TABLE 25 -- IN VITRO INFLUENCE OF THYROXINE ON RESPIRATION OF NORMAL MALE RAT LIVER SLICES; KREBS-RINGER PHOSPHATE MEDIUM; GLUCOSE SUBSTRATE

Animal No.	gm body wt.	$-Q_{O_2}$/hr/10 mg dry wt.* Final molar conc. of thyroxine 0	10^{-6}	10^{-5}	10^{-4}
96	203	42.7 ± 1.9	40.1 ± 1.1	46.4 ± 0.9	42.0 ± 2.1
97	184	57.3 3.6	56.4 0.4	59.8 3.4	58.3 1.7
99	209	54.0 0.7	55.7 2.8	50.0 1.6	53.6 0.8
100	163	36.8 4.3	35.0 0.9	39.0 4.0	35.9 1.4
101	190	47.4 0.5		41.0 2.3	
102	170	50.1 1.5		49.1 0.5	
103	216	63.2 3.8		60.3 2.0	
Average	191	50.2	46.8	49.4	47.4

* duplicate measurement with average deviation

of 10^{-5} and 10^{-4} M triiodothyronine (Table 24). As concluded by Barker (1951), *in vitro* presence of L-thyroxine at concentrations of 10^{-6} to 10^{-4} M does not increase respiration of surviving tissue (male rat liver slices) (Table 25).

Throughout previous experiments some observations were made on *in vitro* influence of these hormones on certain enzyme systems. In the case of oxidative phosphorylation by liver residues (α-ketoglutarate substrate), thyroxine is without any detectable effect. However, when one averages the averages of each pair of P:O ratios for all three experiments (Table 7) the presence of triiodothyronine gives 3.10 $\pm$ 0.16 (with average deviation) compared to 2.66 $\pm$ 0.05 for the control value and 2.59 $\pm$ 0.50 when thyroxine is present. Both hormones were at 1.1×10^{-5} M levels. Neither had any noticeable effect on a lactating mammary gland "washed residue" (prepared in presence of EDTA) oxidation of α-ketoglutarate (Table 21).

DISCUSSION

The R. Q. data are in good agreement with Folley and French's values of 0.83 for the 20th day of pregnancy, 1.0 for the first day of lactation, and about 1.6 for the 8th and 15th day of lactation (glucose substrate) (Folley and French, 1949b). These authors suggest that the R. Q. above 1 during lactation reflects the net fat synthesis of the tissue but this interpretation has doubtful relation to the observation of the very high R. Q. of the virgin rat's mammary gland. Furthermore, the apparent aerobic glycolysis tended to be considerably lower relative to respiration than Folley and French (1950) reported. The rise of glycolysis in involuted tissue reflects a relative predominance of these processes over the respiratory system at a time of alveolar regression.

Since the rat milk secretion gradually increases well beyond 9 days postpartum (Brody and Nisbet, 1938) when the R. Q. tends to plateau, there seem to be metabolic processes mediating the secretion of milk which are not exclusively reflected in the respiratory quotient (Fig. 5).

The more noticeable scattering of data from tissue taken from animals in late lactation and post-lactation is probably due to varying degrees of self weaning and suggests caution in metabolic work involving the use of mammary tissue from such animals.

The rise in oxyen consumption and "metabolic" acid production per unit nitrogen during pregnancy has special interest. This is a time when epithelial cells gradually become relatively more frequent than fat and connective tissue cells, and colostrum is not a complicating factor. Furthermore, the mammary gland pad as dissected from the animal also has a gradually increasing total nitrogen content during this same phase (Kirkham and

Terner, 1953; Fig, 2). During advancement of pregnancy the increasing nitrogen also has, per unit, an increasing metabolic activity associated with it. Since the DNA (presumably a fair index to cell numbers) of the tissue increases during this time (up to mid-pregnancy), it is difficult to be certain whether the metabolic increase reflects greater activity of epithelial cells or a mere relative increase of them above less active structures.

With the demonstration that mammary gland preparations (residues from homogenates and presumably whole homogenates) were much more active in oxidation of some Krebs cycle intermediates when prepared in presence of EDTA, it would seem that many of the earlier studies reported here and in the literature might reflect activities far below those actually possible to observe. Furthermore, studies reported here involving homogenates or fractions thereof were made at levels of reconstituted factors which were not demonstrated to be optimum. However, their final concentrations were near or above values frequently reported in the literature for similar studies on other tissues.

Studies on oxidative phosphorylation were made with intention of gaining some insight into the mechanism of "biological energy" generation by this highly active tissue. It seemed most reasonable to anticipate that ATP would be the molecular donor of energy for both tissue maintenance and synthesis. Hence, efforts were made to demonstrate the incorporation of energy resulting from oxidation, especially Krebs cycle intermediates, into this nearly omnipotent molecule. Speculation that oxidative phosphorylation could be intimately associated with synthetic activity has been given some experimental basis by the observation that rat liver mitochondria incoporate radio-phosphate into "phosphoprotein" under conditions which were suitable for active oxidative phosphorylation (Friedkin and Lehninger, 1949). Phosphoserine is a component of casein (Lipmann, 1933) and has been found to be a component of the "phosphoprotein" fraction. This phosphoamino acid had a much greater specific activity than the whole fraction following isolation from cells which had been incubated in presence of radio-phosphate (Kennedy and Smith, 1954).

The conclusion that whole liver homogenates could "repair" inability of similar mammary gland preparations to carry out oxidative phosphorylations is based upon data in Table 11. Considering only Experiment 8, the expected oxygen consumption upon mixing the two tissue homogenates was 3.4/2 + 10.8/2 or 6.9 μ atoms of oxygen. The expected value for net μ M P was -(0.5 - 16.1)/2 or -7.8 μ M which would give an expected P:O ratio of 7.8/6.9 or 1.13. The experimental ratio was 1.5. However, from Fig. 7 it was found that decreasing the liver enzyme concentration resulted in less efficient phosphate uptake, which would tend to make the calculated expected phosphate uptake below 7.8 —a factor that exaggerates further the

empirical discrepancy. A liver cell-free, particulate-free (supernatant preparation was not tested for this ability to repair absent phosphorylative activity in such mammary gland preparations.

In phosphorylating systems involving HDP and presumably nearly complete blockage of enolase (since fluoride, magnesium, and phosphate ions were present) it is reasonable to conclude that these mammary glands were actively dismutating HDP as concluded by Terner (1954). This is presumably by way of diphosphoglyceric acid and the ATP trapping system since the absence of hexokinase obliterated (or nearly so) net inorganic phosphate uptake (Table 15). The same was also true for the absence of the fluoride ion which probably was giving extensive protection against phosphatase (including ATPase) activity. In one case, absence of both fluoride and hexokinase resulted in much more inorganic phosphate release than when either alone was absent (Exp. 22; Table 11). DNP at 10^{-4} M levels was without definite alteration of the course of reactions but IAA eliminated phosphate uptake and decreased oxygen consumption well below that rate observed in absence of HDP. Such endogenous rates were appreciable (Table 18). But since the studies on rat mammary gland slices showed low aerobic acid production, it became unreasonable to conclude that such "glycolytic" reactions were the exclusive pathways of ATP synthesis. The probable accumulation of phosphoglyceric acid was not studied. These studies are in general agreement with similar ones recently reported by Terner (1952, 1954).

Although cell-free guinea pig mammary gland tissue preparations have been reported to give oxidative activity in presence of Krebs cycle intermediates (Moore and Nelson, 1952), such properties were not observed in preparations from the rat when the medium was a salt solution (also reported by Terner, 1954) or isotonic sucrose. Neither was it observed with tissue from the goat, rabbit, or cat, although no persistent effort was made to do so (Table 14). With stabilization by EDTA, oxidation of some Krebs cycle intermediates was accompanied by inorganic phosphate uptake.

Studies of thyroid hormones, *in vitro,* especially 3, 5, 3'-L-triiodothyronine which is physiologically more active than thyroxine on respiration of surviving tissue slices, were almost completely negative. The one possible exception was triiodothyronine's increase of the respiratory coefficient from a control value of 50.5 to 56.2 at 10^{-5} M level (Table 22). Similar measurements on hypothyroid animals were made with the assumption that the thiouracil effect would only be by way of depressed thyroid secretion.

The few experiments which showed that triiodothyronine *increased* the P:O ratio (thyroxine giving a slight or no reduction) is not in accord wth Lardy's postulation that thyroxine depresses efficiency of high energy

phosphate bond formation with, however, a net increase in such synthesis. This triiodo compound has been said to be more potent than thyroxine in increasing succinate oxidation of a rat heart homogenate preparation (Wiswell and Asper, 1953), which was not observed in a residue of rat mammary gland which was oxidizing α-ketoglutarate (Table 21).

SUMMARY AND CONCLUSIONS

1. The uniqueness of the mammary gland as a material for experimental studies of growth, secretion (work), and aging (involution) was discussed, especially in terms of metabolic control and the possible role of hormones.

2. Carbohydrate, fat, phosphorus, and nitrogen intermediary metabolism of the mammary gland and its direct hormonal alteration was discussed.

3. Mechanism of thyroid hormones' (in part, galactopoietic agents) action was discussed.

4. *In vitro* respiration, aerobic glycolysis, and "metabolic" carbon dioxide production (R. Q. calculated) per unit nitrogen of rat mammary gland tissue in various physiological states was studied in detail. It was found that these processes were closely related to the physiological state.

Both oxygen consumption and "metabolic" carbon dioxide production increased through the first half of pregnancy, increased dramatically following parturition (the latter greater than the former), and fell upon tissue involution.

Aerobic acid production was low throughout the reproductive cycle except for some increase during the latter part of pregnancy, early lactation, and especially upon involution where its index approached the index for respiration.

Although some very high R. Q. values were observed on tissues from virgin animals, they were in general below 1 throughout pregnancy, following which they increased to ca. 1.7 in 8 to 10 days of lactation. In general the R. Q. was below 1 for tissue which had involuted.

5. Adenosine triphosphate synthesis was studied in lactating rat mammary gland homogenates and fractions thereof. Whole homogenates (isotonic sucrose medium) and "washed residues" (saline medium) were unable to take up inorganic phosphate in presence of a hexokinase system for trapping adenosine triphosphate, and some members of the Krebs cycle. The system oxidized succinate to some extent. Some other members of the Krebs cycle were not oxidized or were only slowly oxidized by such preparations. Similar preparations from rat liver did carry these enzymic activities.

This mammary gland system (aerobic, fluoride ions present) was effective in taking up inorganic phosphate in presence of hexose diphos-

phate. Monoiodoacetic acid obliterated the phosphate uptake and depressed oxygen consumption below the endogenous level. This suggests that the mammary gland dismutates hexose diphosphate into trioses.

Sedimentable residues from homogenates were found to oxidize some Krebs cycle intermediates when prepared in presence of the chealating agent, ethylenediaminetetraacetic acid. In presence of α-ketoglutarate oxidative phosphorylation was demonstrated and, in which case, 2,4-dinitrophenol (10^{-4} M) depressed the P:O ratio.

6. The direct *in vitro* influence of 3, 5, 3' -L-triiodothyronine, and to a lesser extent L-thyroxine, on some metabolic processes was studied.

Q_{O_2} of normal rat liver slices incubated one hour in presence of triiodothyronine (10^{-5}M) was increased from 50.5 (control value) to 56.2. Other concentrations (10^{-8} to 10^{-4} M) produced less or no effect, as was true for similar studies made on such tissue from hypothyroid animals. No effect (10^{-6} and 10^{-4} M) could be found on glucose, succinate, or pyruvate oxidation by rat kidney slices. Other negative findings were recorded.

P:O ratios of rat liver homogenate residues in presence of α-keto glutarate (three experiments) were *increased* by *in vitro* presence of triiodothyronine (1.1×10^{-5} M). The same concentrations of thyroxine were essentially without effect.

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ABBREVIATIONS

AMP—Adenosine monophosphate, muscle adenylic acid
ATP—Adenosine triphosphate
A-V—Arterio-venous
Co A—Coenzyme A
Co I—Coenzyme I, cozymase, diphosphopyridine nucleotide, DPN
Co II—Coenzyme II, triphosphopyridine nucleotide, TPN
DNA— Desoxyribonucleic acid
DNP —2, 4-dinitrophenol
EDTA —Ethylenediaminetetraacetic acid
F-6-P—Fructose-6-phosphate
G-1-P—Glucose-1-phosphate
G-6-P—Glucose-6-phosphate
HDP—Hexose diphosphate, fructose-1-6-diphosphate
OAA—Oxalacetic acid
PGA—Phosphoglyceric acid
6-P-G—6-phosphogluconic acid
RNA—Ribonucleic acid, pentosenucleic acid, PNA
R-5-P—Ribose-5-phosphate