in greater amount than pyruvate, and other α -keto acids are also present in small amounts. Kulonen *et al.* (1952) reported a mean daily excretion in five subjects of 16.9 mg. of pyruvate and 14.0 mg. of α -oxoglutarate. The level of activity of the subjects is not mentioned, but it seems likely that the high pyruvate excretion may have been the result of exercise, since it has been shown by Johnson & Edwards (1937) and by McArdle (unpublished work) that the excretion of pyruvate is greatly increased after strenuous exertion. A point of some interest that is being further investigated is the higher excretion rate of α -oxoglutarate in women.

SUMMARY

1. A simplified procedure is described for the separation of the 2:4-dinitrophenylhydrazones of α -keto acids by paper chromatography, and for the quantitative estimation of the separated hydrazones of pyruvate and α -oxoglutarate.

2. Values are given for pyruvate and α -oxoglutarate in post-absorptive blood from healthy resting human subjects, in cerebrospinal fluid and in urine.

3. The rate of excretion of α -oxoglutarate in urine is greater in women than in men.

This work was carried out on behalf of the Medical Research Council. I wish to thank Professor R. H. S.

Thompson and Dr M. F. S. El Hawary for help and advice. I am greatly indebted to Miss H. Pels and Miss F. Crew for their skilled assistance.

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Studies on the Particulate Components of Rat Mammary Gland

1. A METHOD FOR DETERMINING THE COMPOSITION OF THE RETAINED FLUID

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(Received 15 November 1956)

The mammary gland of the rat as dissected from the animal is a complex system containing not only mammary tissue itself but also the secreted fluid retained in the alveoli and duct system of the gland (colostrum in pregnancy, milk during lactation). Tissue suspensions of rat mammary gland prepared at different stages of the lactation cycle contain a variable quantity of this retained fluid which, in the later stages of lactation, may account for up to 60 % of the gross weight of the gland (Folley & Greenbaum, 1947). As the retained fluid has neither the same chemical constitution nor the same enzymic activity as the parent mammary tissue it is essential, in quantitative studies on mammary tissue, to correct analyses on the total gland for the amount of

retained fluid found in the wet tissue when it is excised from the body.

A method has been published for the estimation of retained milk in suspensions of rat mammary tissue (Folley & Greenbaum, 1947) in which the percentage of lactose in milk obtained from lactating rats was determined and then used to calculate the percentage milk retained in tissue suspensions at all stages of the lactation cycle. While this method gave reasonable values for milk retention during lactation, where the percentage of lactose in milk was shown to be approximately constant, it was almost certainly in error when applied to rats at other stages of the lactation cycle such as late pregnancy, shortly after parturition or early in

involution. The percentage of lactose in the secreted fluid is different in late pregnancy compared with lactation. Colostrum has, in general, a lower lactose content than milk of the same species (Woodman & Hammond, 1923; Davies, 1936). The method proposed by Folley & Greenbaum (1947) is limited to periods where samples of retained fluid can be obtained for lactose analysis and so is not applicable to periods other than lactation. The present paper describes an indirect method for determining the lactose content of secreted fluid at certain stages when direct analysis is not possible.

In any mammary-tissue suspension, it is obvious that as the volume of retained fluid/gram increases so the tissue content/gram must decrease by a corresponding amount. If the amount of retained fluid and the tissue content could both be measured without error, the relation between the two measurements would be linear over the entire ranges 0–100 % (retained fluid) and 100–0 % (tissue). Since these quantities cannot, however, be measured directly it is necessary to estimate them indirectly. For any one stage in the lactation cycle the lactose content of the retained fluid is assumed to be constant, and measurements of lactose should therefore be proportional to the amount of retained fluid. Similarly, if some quantity proportional to the tissue content can be measured, the relation between this quantity and the lactose content would be linear.

It is not generally possible to obtain a direct estimate of the tissue present in mammary-gland suspensions in terms of nitrogen. The total nitrogen of the suspension contains a contribution resulting from the presence of nitrogen-containing compounds (casein, albumin, etc.) in the retained fluid; this contribution cannot be determined except in lactation. Some constituent, other than nitrogen, which is proportional to the tissue present must be used to obtain the correlation between tissue and lactose.

In the absence of mitosis, deoxyribonucleic acid (DNA) has been shown to be constant for each type of cell in a great variety of tissues (see Vendrely, 1955). In the absence of marked alterations in polyploidy or of multinucleate cell numbers, the DNA content of a mitotically quiescent tissue is directly proportional to the number of cells in that tissue. No alterations in the numbers of polyploid or multinucleate cells have been reported in the rat mammary gland. Sternberg (1950) has shown that there is no significant amount of either ribonucleic acid (RNA) or DNA in rat milk. Finally it will be shown in the second paper in this series (Greenbaum & Slater, 1957*a*) that, although a burst of mitotic activity occurs in the mammary gland some 30 hr. *post partum*, the period during which cell division occurs is sharply defined and is completed in a

relatively short period; 24 hr. before, or after, this period of mitotic activity the tissue is mitotically quiescent. In a group of animals of the same functional status, the DNA contents of mitotically quiescent tissue samples are proportional to the tissue contents of the samples (see De Robertis, Nowinski & Saez, 1955). In more precise terms, Hoffman (1953) states that the two major parameters governing the frequency distribution of cell size in a tissue sample are the ages of the cells and the mitotic rate. Thus, in a group of animals with identical histories, and where mitotic activity is low, the frequency distributions of cell size are practically identical, so that in a large sample of tissue the tissue present is proportional to the number of cells present and hence to the DNA content of the sample.

These considerations apply to the mammary gland during the lactation cycle except at three periods: (a) during early pregnancy when rapid growth is occurring, (b) some 30 hr. after parturition when, for a brief period of some 12 hr., a burst of mitosis occurs, and (c) possibly during involution. It is therefore possible to use the level of DNA in a sample as a measure of the amount of tissue present in that sample except during the periods mentioned above. With DNA as a measure of the amount of tissue present, it is possible to use the relationship described above and hence to derive the percentage of lactose in the retained fluid. This figure, in turn, permits the calculation of the percentage of retained fluid in a tissue suspension. The indirect method described here is applicable to the estimation of retained fluid in the gland at the physiologically important times of late pregnancy, at parturition and during most of lactation.

METHODS

Definitions. (a) The term 'retained fluid' refers to the secretion which is retained in the ducts and alveoli of the excised gland (colostrum in pregnancy, and milk in lactation). (b) The term 'total nitrogen' refers to the nitrogen content of the whole mammary-tissue suspension. This includes the nitrogen content of the mammary tissue itself and also the nitrogen of the retained fluid. (c) The term 'tissue nitrogen' refers to the nitrogen contribution to the mammary suspension from the mammary tissue alone.

Animals. Adult, female albino rats of approximately 200 g. were used throughout this investigation. With rats needed for the 20-day pregnancy values, male rats were introduced into a cage of virgin females for one night only; the length of the ensuing pregnancy was measured from the day of removal of the males.

Rats were killed by dislocation of the cervical vertebrae. The abdominal glands were quickly dissected from the skin and placed in ice-cold 0.25 M-sucrose. After weighing, the glandular tissue was finely chopped with scissors, homogenized in a loose-fitting all-glass Potter-Elvehjem homogenizer (Potter & Elvehjem, 1936), and then re-homogenized

in a tight-fitting homogenizer having a polythene piston with a stainless-steel shaft. The suspension was diluted with cold 0.25M-sucrose to give a 1:10 tissue suspension and then strained through muslin to remove intractable connective and fatty tissue. The above procedure, finally adopted after trying several variations such as a stainless-steel Potter-Elvehjem homogenizer and a Folley & Watson (1948) top-drive blender, is very similar to that developed by Popják & Tietz (1954).

Estimation of lactose in mammary tissue. The method developed by Folley & Greenbaum (1947) for the estimation of lactose in mammary suspensions is satisfactory and has been used in this investigation.

Estimation of DNA in mammary-tissue suspensions. The DNA content of mammary-tissue suspensions was determined throughout this investigation by the diphenylamine reaction of Dische (1955). The procedure adopted was to extract the nucleic acids from 2 ml. of suspension by the procedure of Schneider (1945), and then to heat 2 ml. of extract with 4 ml. of diphenylamine reagent (Dische, 1955) for 15 min. on a boiling-water bath. After cooling, the colour was measured on a Unicam SP. 500 spectrophotometer at 600 m μ . In order to make the values comparable with other figures in the literature the DNA values have been calculated as deoxyribonucleic acid phosphorus (DNA-P).

Fig. 1. Calculation of the lactose content of rat milk. *A*, At the 20th day of pregnancy; *B*, figures taken for all stages of lactation; *C*, on the 2nd day of mammary involution. The lactose content is calculated (as g./100 g. of milk) from the equation of the regression line.

RESULTS

The results of the determinations of lactose and DNA have been grouped into sets of distinct functional status and are given in Table 1. The regression equation relating the lactose content with the DNA-P content of the mammary suspensions are also included.

Fig. 1 shows the individual lactose and DNA-P values used in calculating the regression equations given in Table 1. The percentage of lactose in the retained fluid for each stage of the lactation cycle investigated was derived from the equation of the regression line as mentioned above. It was not possible to separate regressions corresponding to the third, tenth or eighteenth day of lactation so that a constant lactose content in milk throughout lactation is indicated by this method. The values for the percentages of lactose in retained fluid were then coupled with experimentally determined lactose contents for each mammary suspension to calculate

Fig. 2. Calculation of the nitrogen content of rat milk and mammary tissue. *A*, At the 20th day of pregnancy; *B*, at the 3rd day of lactation; *C*, at the 18th day of lactation; *D*, on the 2nd day of mammary involution. The nitrogen content of milk (g./100 g. of milk) is obtained from the regression line as the abscissa corresponding to 100% retained fluid on the ordinate. The nitrogen of the mammary tissue (g./100 g. of milk-free tissue) is obtained from the regression line as the abscissa corresponding to 0% retained fluid on the ordinate.

Table 1. Relationship between lactose and DNA-P: the lactose content of retained fluid, and the level of retained fluid in rat mammary gland

Status	DNA-P		Regression of lactose content on DNA-P content		Lactose content of retained fluid (g./100 g. of fluid)	Lactose g./100 g. wet wt. of gland (range)	Mean percentage of retained fluid in fresh gland
	No. of animals	mg./100 g. wet wt. of gland	No. of animals	Equation			
Virgin	6*	4.8 ± 0.1†	—	—	—	—	—
Pregnant (20 days)	11	14.7 ± 2.2	8	Lactose = 1.85-0.041 (DNA-P)	1.85	1.21 (0.60-1.80)	66
Lactating (3 days)	10	22.9 ± 2.3	20	Lactose = 2.55-0.027 (DNA-P)	2.55	1.95 (1.52-2.33)	76
Lactating (10 days)	6	20.7 ± 1.9				1.98 (1.85-2.09)	77
Lactating (18 days)	9	25.2 ± 2.6				1.88 (1.39-2.39)	72
Involuting (2 days)	9	17.5 ± 3.1	6	Lactose = 5.11-0.175 (DNA-P)	5.11	2.35 (0.80-3.70)	46

* Analyses on two pooled samples; three animals were used per sample.

† In all tables the values given are group means ± standard error of the mean.

Table 2. Relationship between the level of retained fluid and total nitrogen: tissue protein, and the protein of the retained fluid in rat mammary gland

Status	Total nitrogen		Regression of the percentage of retained fluid (a.r.) on the total nitrogen content of mammary-tissue suspensions		Tissue protein*		Protein content of retained fluid (g./100 g. of fluid)
	No. of animals	g./100 g. wet wt. of gland	No. of animals	Equation	g./100 g. wet wt. of gland	g./total gland	
Virgin	6†	0.266 ± 0.020	—	—	1.70	0.043	—
Pregnant (20 days)	10	0.504 ± 0.037	14	% R.F. = 16.467 (Total N) - 8612 + 0.016	1.17	0.054	3.4
Lactating (3 days)	9	1.083 ± 0.085	9	% R.F. = 118.4 - 42.5 (Total N)	4.28	0.193	2.8
Lactating (10 days)	6	0.921 ± 0.076	—	—	3.91†	0.238†	—
Lactating (18 days)	8	1.513 ± 0.050	10	% R.F. = 177.4 - 69.96 (Total N)	4.54	0.254	7.1
Involuting (2 days)	6	1.207 ± 0.117	5	% R.F. = 129.3 - 59.95 (Total N)	7.45	0.491	3.1

* Calculated from the mean percentage of retained fluid (a.r.) in Table 1.

† Analyses on two pooled samples; three animals were used per sample.

‡ Based on a protein content of milk-free gland equal to the average of the third and eighteenth day lactation groups.

the percentage of retained fluid in each suspension. The percentage of lactose in the retained fluid at each stage of the lactation cycle, as derived from the regression lines, and the mean percentages of retained fluid in the groups of mammary suspensions are given in Table 1.

Determination of the contributions by tissue nitrogen and retained-fluid nitrogen to the total nitrogen of mammary-gland suspensions

The total nitrogen content of suspensions of rat mammary gland at the six stages of the lactation cycle considered are given as mean values in Table 2.

The total nitrogen of the suspension is the sum of two contributions: the nitrogen content of the gland tissue and the nitrogen content of the retained fluid present. For the stages of late pregnancy, early lactation and involution, the same difficulties as those affecting direct lactose analysis operate to prevent direct analysis of the nitrogen content of the retained fluid. However, the basic assumption used in the lactose intercept procedure can be used to overcome the difficulties at the periods studied. If the same assumption is made with respect to the nitrogen of the gland as was made for the lactose, i.e. that at each particular stage of the lactation cycle both the retained fluid and the tissue have a characteristic composition, then the following equation can be derived.

If R_i represents the fraction of fluid retained in the gland, T_i the total protein in the mammary-gland suspension, t the percentage of protein in mammary tissue, m the percentage of protein in retained fluid, then the identity:

total protein of the gland = tissue protein
+ retained fluid protein
can be written in the form

$$T_i = (1 - R_i) t + R_i m,$$

so that

$$T_i = R_i(m - t) + t.$$

If the basic assumption that t and m are characteristic constants is valid, a graph plotting T_i against R_i will be linear. The constant t will give the percentage of protein in mammary tissue and the value of T_i when R_i equals 1 is identical with the percentage of protein in retained fluid.

The individual determinations of the total nitrogen of the mammary-gland suspensions are plotted against the calculated percentage of milk retention in Fig. 2 for the stages investigated. The calculated regression equations of the percentage retained fluid on total nitrogen are given in Table 2, which also includes the values calculated for the percentage protein content in tissue and retained fluid. No regression line could be obtained with the tenth day lactating group, where the retained fluid contents were too closely grouped to obtain a reliable estimate.

DISCUSSION

The method proposed here for the indirect analysis of retained fluid at periods of the lactation cycle where direct analysis is not possible is based on several basic assumptions whose validity will now be considered.

First, it is assumed that at each stage of the lactation cycle studied a characteristic composition exists both for mammary tissue and for retained fluid, i.e. that there would be little difference in composition and properties between individuals of each group of animals studied. This assumption seems reasonable, as none of the periods studied involved periods of rapid growth or very rapid changes in synthetic activity. Some direct evidence can, however, be derived from an examination of the individual standard errors of the means for various components of the mammary gland given in the next two papers (Greenbaum & Slater, 1957*a, b*). The small standard errors of the means of every component examined, at each stage of the lactation cycle investigated, indicates that members of each group do not depart significantly from a mean value identifiable as a group constant.

The second assumption was that, at the periods studied, the DNA-P content of a mammary-tissue suspension was directly related to the mammary tissue present. This assumption has been tested for the lactation period. Samples of rat milk, obtained after the injection of oxytocin, were analysed for total protein and lactose. From these values for milk composition, and the lactose and total nitrogen contents of the mammary suspensions from lactating rats, it was possible to calculate the contributions to the total-suspension nitrogen from the mammary-tissue nitrogen and the retained-fluid nitrogen respectively. The assumption that tissue nitrogen (i.e. tissue protein) is directly related to the DNA-P content of the sample can then be tested by plotting the tissue-nitrogen values against the DNA-P contents. Fig. 3 shows the relationship between DNA-P and tissue nitrogen obtained in this way. From the variability of milk composition reported by Cox & Mueller (1937) it can be seen that the assumption relating DNA-P to tissue nitrogen appears justified at least for the lactation period. It is not possible to verify the assumption for the periods of pregnancy and mammary involution as adequate samples of retained fluid for direct analysis cannot be obtained. However, the verification of the assumption in lactation makes its application to other stages of the lactation cycle not unreasonable, particularly as mitotic activity is rarely found in any of the stages considered.

The basic argument that DNA-P and lactose are linearly related is a corollary of the two preceding assumptions. Independently the argument seems

justified by consideration of the graphs shown in Fig. 1A-C. The linearity of the data in the graphs was tested by fitting both a straight line and a quadratic curve to the data. In no case was the variance about the line significantly greater than that about the curve, indicating that the data were not inconsistent with linearity.

From the figures obtained by the direct analysis of milk samples collected during lactation, it is possible to check the values derived by the 'intercept procedure' for the lactation groups. Table 3 shows a comparison between the values obtained by direct analysis of rat milk for lactose and total protein and the corresponding values calculated by the intercept procedure. The protein values refer to the group at the eighteenth day of lactation. The values found by direct analysis differ from those given by Cox & Mueller (1937) and Folley & Greenbaum (1947), but the difference is probably due to the different strain and diet used, both of which have a considerable effect on milk composition (Cox & Mueller, 1937). It can be seen from Table 3

that the intercept method and direct analysis give closely similar results both for lactose and total protein. The application of the intercept method to the other periods of the lactation cycle studied, where direct analysis is not possible, therefore seems justifiable.

Milk lactose and protein

The results given in Table 1 show that the lactose content of colostrum is smaller than for milk, and that the lactose content of the retained fluid increases in early involution. The rise in the lactose content of the retained fluid over parturition is in agreement with previous work on all other species examined (Davies, 1936).

The rat appears to differ from the larger mammals, however, in the changes in protein content of the retained fluid over the period of parturition. Whereas the general picture is that of a high level of protein (mostly albumins and globulins) in colostrum and a much lower level of protein (mostly casein) in milk, the rat appears to show only a slight fall in the protein content of the retained fluid over parturition, but an increase in the protein content of milk from the third to the eighteenth day of lactation (Table 2). From the data available it is not possible to decide whether the increase in protein content is a rapid one levelling out to a plateau early in lactation, or is the result of a sustained rise throughout the lactation period. The necessity for correcting analyses on rat mammary tissue for the presence of retained fluid has already been stressed.

The question arises (i) whether to use the experimentally found values for the lactose content of retained fluid where this is possible (i.e. in lactation) and the indirectly calculated lactose values for other groups (i.e. pregnancy and involution); or (ii) to use indirectly calculated values for all groups considered. Although experimentally determined values are to be preferred to calculated values, the small difference between the experimental and calculated figures for the lactation period (Table 3) is not sufficient to outweigh the advantage gained by better comparisons between groups when the results have all been calculated by the same procedure. We have therefore used calculated values for the percentage of lactose in retained fluid to

Fig. 3. Relation between mammary-tissue nitrogen and DNA-P.

Table 3. Direct analysis of milk, and values derived by the 'intercept procedure' for rats in lactation

	Lactose* (g./100 g. of milk)			Protein† (g./100 g. of milk)		
	No. of animals	Mean	Standard error of estimate	No. of animals	Mean	Standard error of estimate
Intercept-method	20	2.55	0.14	10	7.1	1.5
Direct analysis	7	2.45	0.30	4	7.6	0.5

* Average for animals taken at various stages throughout lactation.

† Average for animals at the eighteenth day of lactation only.

convert the experimentally determined lactose of the whole homogenate into percentages of retained milk. We believe that, although the absolute magnitudes of the percentages of the retained fluid obtained may be slightly in error, the comparisons between groups of animals will be closer to reality.

Tissue protein

The rapid rise in tissue protein at parturition is of particular interest. The results obtained indicate that the protein content of the retained fluid is sensibly the same for the late-pregnancy and early-lactation groups (Table 2). Since the percentage of retained fluid in the glands does not appear to alter markedly over parturition (Table 1) the rapid increase in total protein can be taken to indicate an increase in cellular material. This is further indicated by the results for tissue protein (Table 2). The rapid increase in tissue protein over parturition can hardly be the result of a cellular hypertrophy, as the available cytological evidence suggests that mammary cells are smaller in early lactation than in late pregnancy (Weatherford, 1929; Jeffers, 1935). The more likely explanation is that an extensive cell division occurs at parturition. Evidence is presented in Part 2 (Greenbaum & Slater, 1957a) that such a mitotic wave does, in fact, occur.

The rapid rise in tissue protein over parturition is followed by a small increase in tissue protein throughout lactation when calculated both as protein/gland and as protein/100 g. of wet tissue. The rise in tissue protein/gland during pregnancy is surprisingly small, but is probably accounted for by the fact that the rats used in this investigation were fully adult females in which the virgin glands had been built up by passage through several oestrous cycles (Kirkham & Turner, 1953). The results for tissue protein in lactation are consistent with the cytological findings of Weatherford (1929) that mitosis is virtually absent from lactating glands although the mammary cells show a progressive hypertrophy. Such a hypertrophy could be the consequence of an increasing water content, a common feature both of synthetically active and rapidly growing cells (see Cowdry, 1955). Although Smith (1956) found no change in the water content of the guinea-pig mammary gland, it should be remembered that the lactation curve of the guinea pig is very different from that of the rat, and Smith's observation does not exclude the possibility of hydration of the rat gland. Indeed, such hydration has in fact been described by Kleiber, Smith & Levy (1943) and by Folley & French (1949b) in rats. Another contribution to the hypertrophy in lactation could be due to an increase in the volume of cellular inclusions, particularly fat. Thus the hypertrophy observed cytologically could be due to either of the above causes as well as to a genuine

cellular expansion concomitant with an overall increase in cytoplasmic material.

The mammary gland undergoes a period of intense chemical and physiological change at and near the time of parturition. In the course of a few hours the entire metabolic pattern of the gland is refashioned. Repeated investigations of the diverse features of the increased glandular activity, illustrated by the rise in R.Q. and Q_{O_2} (Folley & French, 1949a, b), the increase in enzymic activity (Folley & Greenbaum, 1947; Moore & Nelson, 1952; Greenbaum & Greenwood, 1954; Glock & McLean, 1954; Tuba, Orr & Wiberg, 1955), or in the total nitrogen and nucleic acids (Kirkham & Turner, 1953), have led to an appreciation of the magnitude, as well as the rapidity, of these changing patterns at this critical time. The results of this investigation have further illustrated the importance of this period.

SUMMARY

1. The levels of deoxyribonucleic acid, lactose and total nitrogen in suspensions of rat mammary-gland tissue have been determined at intervals during the lactation cycle.

2. A method is described by which the lactose and nitrogen levels of colostrum and milk at periods close to parturition can be determined.

3. Values have been calculated for the contribution by tissue and retained fluid to the total nitrogen of the suspensions. The tissue nitrogen increases slowly during pregnancy, very rapidly over the period of parturition and then slowly again during lactation.

We wish to acknowledge the advice and help of Mr N. W. Please of the Statistics department for assistance in the statistical aspects of this paper. Our thanks are also due to the Agricultural Research Council for an expenses grant to one of us (A.L.G.).

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Studies on the Particulate Components of Rat Mammary Gland

2. CHANGES IN THE LEVELS OF THE NUCLEIC ACIDS OF THE MAMMARY GLANDS OF RATS DURING PREGNANCY, LACTATION AND MAMMARY INVOLUTION

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(Received 15 November 1956)

The deoxyribonucleic acid (DNA) content of any particular type of cell appears to be constant (see Vendrelly, 1955). This constancy of content has been repeatedly demonstrated and deviates only in cells displaying polyploidy, or in multinucleate varieties. Furthermore, isotope-turnover studies indicate that DNA, except in mitotically active cells, is metabolized to a very limited degree (Hevesy & Ottesen, 1943). DNA has, therefore, come to be used as a reference standard directly related to the number of cells present (see Davidson, 1953; Davidson & Leslie, 1950). The value of such a reference standard in studies of the mammary gland, where total nitrogen is not necessarily related to the amount of tissue present, is obvious.

Ribonucleic acid (RNA) is considered to be of importance in the synthesis of protein (see Brachet, 1955). Although the intracellular site of protein synthesis is not fully established, many workers believe it to be in the small-particle fractions of the cytoplasm, the microsomes and submicrosomes (see Brachet, 1955). RNA, however, is widely distributed in the cell and the rapid turnover rate of nuclear RNA (Smellie, McIndoe, Logan, Davidson & Dawson, 1953) indicates that the RNA of the nucleus is probably of considerable importance in protein synthesis.

The mammary gland at parturition undergoes a rapid and intense change in the rate of protein synthesis, lactation being well established within a few hours *post partum*. The amount of casein synthesized/day by an active rat mammary gland is comparable in amount with the parent tissue

present. The mammary gland has, therefore, advantages to offer over other tissues, where the rate of protein synthesis is constant, in studies relating RNA to protein synthesis.

METHODS

Animals. Rats were killed by cervical dislocation and the entire lower abdominal mammary glands quickly removed and placed in ice-cold 0.25M-sucrose.

Seven groups of female albino rats were used: (a) adult virgin rats aged 4–6 months; (b) adult rats, about 200 g. body wt. and 20 days pregnant; (c) adult rats 3, 10 and 18 days *post partum*; (d) adult rats in the second and fourth days of mammary involution. Litters, which in all cases were reduced to 6–8 pups, were weaned 22 days *post partum*.

Homogenizing. After homogenizing by the procedure outlined in the previous paper (Greenbaum & Slater, 1957a) the homogenate was diluted with 0.25M-sucrose to give a 1:10 tissue suspension. Portions of this suspension were used for total-nitrogen determination, nucleic acid determinations and lactose estimation to determine the milk content of the suspensions.

Centrifuging. The mammary gland suspension (30 ml.) was centrifuged to obtain a large-particle (mitochondrial) fraction. The procedure was as follows: after two initial centrifugings at 600 g for 10 min. in a refrigerated centrifuge to remove cellular debris and nuclei, the suspension was centrifuged at 8000 rev./min. for 15 min. at plateau speed in a Servall refrigerated centrifuge (Model SS2). The centrifuge constants for this stage were (Duve & Berthet, 1953): $r_{(\min.)} = 11$ cm.; $r_{(\max.)} = 15$ cm.; $g_{(\text{plateau})} = 10\,900$; temp., 0°. These values give a time integral of about 230 000 g min.

Separation of sediment and supernatant suspension was achieved by decantation. The fatty layer, which was solid at the temperature used in centrifuging, was removed in