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Estimation of Lactose in Rat Mammary-Gland Suspensions

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Excised mammary tissue contains a variable quantity of milk or colostrum retained in the ducts and alveoli. The importance of correcting biochemical analyses of mammary tissue for this variable quantity of retained fluid has been repeatedly stressed (Folley & Greenbaum, 1947; Greenbaum & Slater, 1957*a*). The standard method of measuring the percentage of retained fluid in rat mammary-gland suspensions (Folley & Greenbaum, 1947) is based on the estimation of lactose in clear filtrates of mammary suspensions obtained after precipitation by ferric hydroxide, lactose being estimated by the chloramine-T method of Hinton & Macara (1927). The method of Folley & Greenbaum (1947) gives good recoveries of added lactose from mammary suspensions, and also compares well with other methods of lactose estimation when tried on samples of cow or rat milk (Slater, 1957). An alternative method for the estimation of lactose, based on heating with orcinol (Slater, 1957), is suitable for samples of milk or of mammary-tissue suspensions where lactose is the major carbohydrate present. The orcinol and chloramine-T methods do not agree when used with mammary-gland suspensions (Slater, 1957); the latter method gives higher lactose values. Although protein has been shown to affect chloramine-T values to a much greater extent than orcinol values (Slater, 1957) the discrepancy is not solely due to protein remaining in the ferric hydroxide filtrates.

This paper describes work done to elucidate the discrepancy between the methods which is particularly marked when the mammary tissue is taken from rats in late pregnancy (18–22 days), when the amount of lactose found by the chloramine-T method is approximately four times the value found by the orcinol procedure.

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METHODS

The rats used were black and white adults of the Medical Research Council strain; body weight, 180–200 g. The length of pregnancy was assessed by examination of the foetuses. Animals were killed by cervical dislocation and the abdominal glands were quickly dissected and placed in ice-cold water; tissue suspensions were prepared with a top-drive blender (Folley & Watson, 1948) and were diluted 1:10 with cold water. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with ox serum albumin as standard. Free α -amino acid groups were determined by Folin's method (Hawk, Oser & Summerson, 1947). Free sulphhydryl groups were determined as described by Stadtman (1957). Deoxyribonucleic acid was estimated by the method of Greenbaum & Slater (1957*b*). Ferric hydroxide filtrates were obtained by diluting 5 ml. of the 1:10 suspension with about 15 ml. of water, adding colloidal ferric hydroxide drop by drop until coagulation was complete, making up to 25 ml. with water and filtering. Other methods of precipitation were by (a) trichloroacetic acid [equal volumes of suspensions and cold 10% (w/v) trichloroacetic acid were mixed, kept for 30 min. at room temperature, diluted and filtered]; (b) molybdophosphoric acid [equal volumes of suspension and 5% (w/v) molybdophosphoric acid were mixed, kept for 30 min. at room temperature, diluted and filtered]. Lactose was determined in such filtrates either by the method of Folley & Greenbaum (1947) or by the method of Slater (1957).

RESULTS AND DISCUSSION

The results of lactose estimations performed on ferric hydroxide filtrates are shown in Table 1. It can be seen that in late pregnancy the lactose titre given by the chloramine-T procedure is approximately four times the value given by the orcinol method. In lactation, however, the ratio is only 1.7. It would appear from these results that the chloramine-T procedure is being affected by some interfering substances with reducing properties

which do not similarly affect the orcinol method. It is unlikely that the orcinol procedure is under-estimating, as the experimental procedure is vigorous and involves boiling with strong acid. The interfering substance may arise from mammary tissue itself, being liberated by the process of homogenization, or it may itself be a component of colostrum and milk. The following possibilities were investigated.

Protein

With purified ox plasma albumin it was found that chloramine-T is destroyed linearly by increasing concentrations of protein; 5 mg. of ox plasma albumin added in 10 ml. of solution gave a titration difference equivalent to 3.1 mg. of lactose. Similar concentrations of protein did not affect the orcinol procedure. It is obvious therefore that the presence of substantial quantities of protein in the ferric hydroxide filtrates may well produce a discrepancy between the two methods.

The protein concentration in ferric hydroxide filtrates was therefore investigated at various stages of the lactation cycle. It was found that appreciable quantities of protein material as determined by the method of Lowry *et al.* (1951) were present in the ferric hydroxide filtrates. The mean value for filtrates obtained from rats in late pregnancy was 235 ± 22 mg. of protein/100 g. of original tissue (nine animals). The calculated apparent lactose titre in the chloramine-T reaction resulting from this level of protein in the filtrates is 0.15 g. of lactose/100 g. wet wt. of tissue. Since the discrepancy between the chloramine-T and the

orcinol procedures is approximately 0.4 g. of lactose/100 g. of tissue (Table 1, column 5), it would appear that the presence of protein in the filtrates accounted for about 40% of the total discrepancy. The concentration of protein in mammary-gland filtrates was greatly decreased by saturating the filtrates with ammonium sulphate, filtering and estimating protein in the new filtrates. Precipitation of homogenates of mammary glands with trichloroacetic acid produced filtrates with a low protein content (approximately one-sixth of the protein concentration found in ferric hydroxide filtrates); protein was virtually absent in filtrates prepared with molybdophosphoric acid (Table 2) (cf. Garbach, Dedic & Philippi, 1955).

Sulphydryl groups

Free sulphydryl compounds were investigated for their action on the chloramine-T reaction. Ergothioneine, cysteine and glutathione reduce chloramine-T very rapidly. The rate of reaction is virtually independent of pH between pH 4 and 10, but is decreased if potassium iodide is added during the incubation with chloramine-T. Results for glutathione, which is typical of these compounds, are shown in Fig. 1.

The mean free sulphydryl-group content of ferric hydroxide filtrates was $88 \pm 12 \mu$ -equiv. of SH/100 g. wet wt. of tissue. With the value obtained for the interaction between chloramine-T and glutathione (Fig. 1) such a concentration of sulphydryl groups in the mammary filtrates would lead to an apparent lactose titre in the chloramine-T

Table 1. [Lactose estimations by the chloramine-T and orcinol methods on rat mammary-gland filtrates

Filtrates were prepared with ferric hydroxide as protein precipitant. The numbers in parentheses are the numbers of animals used; mean values are given $\pm$ S.E.M. The values in columns 4 and 5 are direct comparisons obtained from assays on samples of the same filtrate.

Status	Lactose (g./100 g. of tissue)		Column 3 Column 2	Column 3 - column 2
	Orcinol method	Chloramine-T method		
Virgin	0.12 (6)	—	—	—
Late pregnancy	0.16 ± 0.02 (16)	0.58 ± 0.06 (16)	3.70 ± 0.39 (16)	0.42 ± 0.07 (16)
Late lactation	0.86 ± 0.08 (10)	1.47 ± 0.13 (10)	1.75 ± 0.40 (10)	0.61 ± 0.08 (10)
2-Days involuting	0.92 ± 0.17 (5)	2.35 ± 0.41 (6)	—	—

Table 2. Protein and lactose in filtrates obtained from a late-pregnancy mammary-gland suspension

Method of precipitation	Protein in filtrates (mg./10 ml. of filtrate)	Lactose in filtrates (mg./10 ml. of filtrate)	
		Orcinol method	Chloramine-T method
Ferric hydroxide	1.84	0.59	1.82
Trichloroacetic acid	0.30	0.61	1.10
Molybdophosphoric acid	0.08	0.62*	— †

* Correction was made for decreased colour development in the presence of molybdophosphoric acid.

† Molybdophosphoric acid interferes in the chloramine-T reaction.

reaction of 0.07 g./100 g. of tissue. This is approximately 15% of the total discrepancy between the chloramine-T and orcinol procedures in late pregnancy (Table 2, column 5).

Amino acids

Amino acids interact to a limited extent with chloramine-T in the presence of potassium iodide at pH 10, i.e. under the conditions used in the assay procedure for lactose with the chloramine-T reagent. In the absence of potassium iodide the reaction was much more extensive. Fig. 1 illustrates the results obtained with leucine with and without potassium iodide addition during chloramine-T incubation. As protein interferes in amino acid-estimation procedures, the amino acid results have been corrected for the concentration of protein present. (This correction was usually about 15% of the overall value.) There was 1.22 ± 0.09 m-equiv. of amino acid nitrogen/100 g. of tissue in ferric hydroxide filtrates during late pregnancy.

Fig. 1. Destruction of chloramine-T by amino acids. 0.005N-Chloramine-T (10 ml.) was incubated with various quantities of amino acids, with and without the addition of 2.5 ml. of 10% (w/v) KI, for 2 hr. at room temperature. At the end of incubation 2 ml. of 2N-HCl was added, and 2.5 ml. of 10% (w/v) KI was added to those flasks incubated in its absence. The liberated iodine was titrated with 0.005N- $\text{Na}_2\text{S}_2\text{O}_3$. Blank values were obtained in the absence of added amino acid, and the ordinate shows the difference between the blank value and the values obtained in the presence of amino acid. Under the conditions used, 1 ml. titration difference corresponds to 0.86 mg. of lactose added during incubation. Glutathione with KI (●), without KI during incubation (○); leucine with KI (▼), without KI (▲); ergothioneine without KI (■).

From the reaction values for leucine and chloramine-T (Fig. 1) it can be calculated that the concentration of free amino acid in ferric hydroxide filtrates during late pregnancy (other than amino acids with free sulphhydryl groups) leads to an apparent lactose titre of 0.10 g./100 g. of tissue. This is approximately 20% of the total discrepancy.

Free amino acids have been reported to interact with chloramine-T (Cristol, 1956; Gerok & Waller, 1956). Both investigations were, however, concerned with the manometric reaction in the absence of potassium iodide. It has been shown in this paper that the amino acid-chloramine-T reaction can also be followed iodometrically, and that both amino acids and sulphhydryl groups interfere with lactose estimations by the chloramine-T procedure of Hinton & Macara (1927). The contributions of the amino acid pool and free sulphhydryl groups in mammary-gland filtrates to the overall discrepancy between the chloramine-T and orcinol methods is difficult to assess accurately mainly because the destruction of chloramine-T by amino acids and sulphhydryl compounds varies with each substance (T.F. Slater, unpublished work). Preliminary experiments, with paper chromatography, have shown that there is a variety of amino acids in mammary-tissue extracts in late pregnancy as reported by Kuretani (1957). On the basis of the results with leucine and glutathione, it would appear that 30–40% of the total discrepancy between the two methods of lactose estimation arises from interaction of chloramine-T with free amino acid and with sulphhydryl groups in mammary-gland filtrates. A further 40% results from the presence of protein in ferric hydroxide filtrates; this can be removed by alternative methods of protein precipitation.

Ion-exchange chromatography

Ferric hydroxide filtrates from late-pregnancy glands were adjusted to pH 9.0 with sodium

Table 3. Protein, free amino acid nitrogen and lactose in ferric hydroxide filtrates of a mammary-tissue suspension obtained by pooling two glands from animals on the twentieth day of pregnancy

Estimations were carried out on the untreated filtrate and on the same filtrate after passage through Dowex-1 resin. Values are expressed as $\mu\text{g./ml.}$ of filtrate.

	Untreated filtrate	Filtrate after passing through Dowex 1
Protein	88	20
Amino acid nitrogen	4.6	1.5
Lactose by chloramine-T	103	43
Lactose by orcinol	56	9

hydroxide and were then passed through a column (10 cm. $\times$ 0.7 cm.²) of Dowex 1 resin (formate form). Approximately two bed volumes of filtrate were passed through the column, and the resulting effluent was rejected. Subsequent effluent was collected and, together with the original mammary filtrate, was analysed for free amino acid nitrogen, protein and lactose. Table 3 shows that not only did the ion-exchange column decrease the protein and amino acid concentrations as expected, but the lactose value also fell. Control experiments showed that lactose was not adsorbed by the column.

Retained-fluid content of mammary tissue in late pregnancy

It seems possible that the carbohydrate estimated by the orcinol procedure in late-pregnancy mammary glands is not primarily lactose, since Kuretani (1957), using paper chromatography, failed to find lactose in extracts of late-pregnancy rat mammary gland. However, Shimizu, Shoda, Kuretani & Ugami (1957), using the method of Malpress & Morrison (1949), reported the presence of lactose in such extracts. On the other hand, lactose is the predominant carbohydrate in lactating mammary gland, although trace amounts of other carbohydrates have been reported in rat milk in early and late lactation (Iwamura, Hidaka & Nonaka, 1956), in human milk (Montreuil, 1957; Malpress & Hylten, 1958) and in rat mammary gland (Trucco & Caputto, 1954).

It is possible that such trace carbohydrates occur in late pregnancy in similar concentrations to those in lactation. If this were true then the 'trace carbohydrates' would constitute the major part of the carbohydrate-reacting component, since the total material reacting as carbohydrate is low in late-pregnancy extracts. Some evidence that the carbohydrate component in such extracts is largely bound to an acidic component comes from the results shown in Table 3; most of the orcinol-reacting material is adsorbed by the anion-exchange resin Dowex 1 under alkaline conditions, which do not affect free lactose. Such an acidic component could be a nucleotide (Wang & Greenbaum, 1960) or neuraminic acid (Trucco & Caputto, 1954). However, although lactose is probably not the predominant carbohydrate measured by the orcinol procedure in late-pregnancy mammary suspensions, the carbohydrate so measured may be a constituent of retained fluid. If this were so then the amount of carbohydrate in a sample of mammary tissue could be taken as an indication of the level of the retained fluid in that sample.

It is possible to decide whether the carbohydrate component in late-pregnancy samples is a con-

stituent of the retained fluid by using the intercept procedure of Greenbaum & Slater (1957*a*). In this procedure any component associated solely with retained fluid would show an inverse correlation with the deoxyribonucleic acid phosphorus (DNA-P) of the sample. It is known that DNA-P is associated solely with the tissue component of rat mammary gland (Greenbaum & Slater, 1957*a*). Such a reinvestigation of the intercept procedure in late-pregnancy mammary tissue was desirable, since previous values obtained with this approach (Greenbaum & Slater, 1957*a*) used the chloramine-T method for estimating lactose, a method shown in this paper to be greatly in error. Estimations of carbohydrate by the orcinol procedure and of DNA-P were made on late-pregnancy suspensions, and the regression of DNA-P on carbohydrate was calculated. With DNA-P expressed as mg./100 g. of gland and carbohydrate as g./100 g. of gland the relevant statistical values were: DNA-P = $11.03 - 6.86[\text{carbohydrate}]$; number of animals = 20; regression coefficient = 0.81; $P \leq 0.001$; intercept on the carbohydrate axis = percentage of carbohydrate in retained fluid = 1.61 g./100 g. of retained fluid. The strong inverse correlation reported between DNA-P and carbohydrate indicates that the carbohydrate component measured by the orcinol procedure is present in the retained fluid of the gland. It is therefore possible to use the carbohydrate concentration of mammary tissue in late pregnancy as a measure of the retained fluid content of the sample.

The value found in late pregnancy for the carbohydrate concentration in retained fluid was 1.61 g./100 g.; this is reasonably close to the value 1.88 g./100 g. found by the chloramine-T method (Greenbaum & Slater, 1957*a*). However, although the apparent carbohydrate concentration in retained fluid is very little altered if the orcinol procedure is used in place of the chloramine-T method, the concentration found in mammary suspensions is very much decreased (Table 1, columns 2 and 3). This finding leads to lower values for the percentage retention of fluid in mammary gland, particularly in late pregnancy, where retention appears to be between 10 and 20% (i.e. 10–20% of the wet weight of the gland is retained fluid). These new figures are more consistent with the cytological appraisals of the gland at this stage by Weatherford (1929) and Dempsey, Bunting & Wislocki (1947) than previous values of approximately 40% given by Greenbaum & Slater (1957*a*). The percentage retention in lactation appears to be about 40% on the basis of the orcinol values for lactose (Table 1, column 2), whereas chloramine-T determinations had previously indicated values of up to 70% (Greenbaum & Slater, 1957*a*).

SUMMARY

1. The chloramine-T and the orcinol procedures for estimating lactose in tissue suspensions of rat mammary gland have been compared during late pregnancy and in lactation. In lactation, the chloramine-T method gives a higher value than the orcinol procedure (1.7-fold); the discrepancy is greater in late pregnancy (3.7-fold).

2. Factors contributing to the discrepancies have been investigated. Protein coming through the precipitation stage, free amino acids and free sulphhydryl groups all interact with the chloramine-T reagent under the conditions of the lactose assay and thus produce an apparent lactose titre.

3. A reinvestigation has been made of the intercept procedure correlating deoxyribonucleic acid phosphorus with lactose in mammary gland. A strong correlation was found and these investigations have led to revision of previously published values for milk retention in mammary gland.

4. The occurrence of lactose in late-pregnancy extracts is discussed. The results suggest that the lactose titre obtained by the orcinol procedure in late pregnancy possibly arises from carbohydrate components other than lactose and present in retained fluid.

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