significance of these findings in relation to fibre formation is discussed.

4. Unsuccessful attempts to use this reaction to establish the mode of linkage of haem to globin in native haemoglobin are described.

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Studies on the Synthesis of Lactose by the Mammary Gland

I. PRECURSORS

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The main object of our work is to throw some light on the intermediary metabolism of the mammary gland, with particular reference to the mode of lactose synthesis. Although a number of workers, using a variety of experimental techniques, have made an approach to this problem, all studies hitherto reported have been primarily concerned with defining the initial blood precursors of the milk sugar, and there is little information on the detailed mechanism of this synthesis. *In vivo* experiments, in which the concentrations of possible precursors in simultaneously drawn samples of arterial and mammary venous blood were compared, have demonstrated a large uptake of glucose when blood passes through the active mammary gland (for references see Folley, 1940, 1949), and it is regarded as reasonably certain that this uptake is far in excess of the energy requirements of the tissue, and is, in fact, an indication that glucose is a precursor of lactose.

This conclusion has been confirmed and amplified by *in vitro* studies on surviving mammary tissues, notably by Grant (1935) and Knodt & Petersen (1945). Grant (1935) succeeded in demonstrating lactose synthesis from glucose by tissue slices from the lactating glands of guinea pigs killed 2–5 days after parturition, and was able to obtain a quantitative estimate of the synthesis by using a lactose-fermenting yeast, *Saccharomyces fragilis*. Knodt & Petersen (1945) claim to have shown lactose synthesis by tissue slices from the lactating udders of cows killed at slaughter, and in their work glucose, glycogen and maltose were found to be equally effective substrates. Lactic acid, on the other hand, was ineffective, a result contrasting with earlier claims based on arterio-venous studies by Graham (1937). Knodt & Petersen (1945) used fermentation techniques (Scott & West, 1936) in determining lactose and, like Grant (1935), were dependent on changes in reduction values following fermentation for their estimation of lactose formed.

It seemed to us that a first step in our work should be a reinvestigation of these findings, using methods for the estimation and detection of lactose which could be regarded as more specific for this sugar than methods ultimately dependent on reduction measurements. This paper gives an account of this initial study.

EXPERIMENTAL

Reagents. Glycogen was prepared from rabbit livers by extraction with trichloroacetic acid and subsequent precipitation with 50% (v/v) ethanol, 80% (v/v) acetic acid and 50% (v/v) ethanol in that order (Good, Kramer & Somogyi, 1933; Bell & Young, 1934). *s*-Collidine was purified according to Partridge (1948).

Sources of tissues. The collection of cow udders from the local abattoir conformed as closely as possible with the procedure adopted by Knodt & Petersen (1945). Glands showing a high degree of milkiness on removal were selected and brought without special cooling direct to the laboratory. The time from slaughter to the start of an experiment was normally 1·0–1·5 hr.

Guinea pig glands were obtained from animals in our own colony. Parturient does were suckled normally by their litters and killed in active lactation on the sixth day post-partum. In these experiments the incubations of tissue started within 30 min. of killing by neck dislocation.

Preparation of tissues. Both cow and guinea pig glands were prepared in the same way. Tissue slices were cut with a Stadie & Riggs (1944) microtome from small blocks of tissue immersed in Krebs Ringer phosphate (KRP) buffered at pH 7·4 (Krebs & Henseleit, 1932; Umbreit, Burris & Stauffer, 1945). The microtome inset had been ground to cut slices 0·5 mm. thick, but owing to the compressibility of mammary tissue the actual thickness of the slices used was variable, ranging from 0·5 to 1·0 mm. The slices were quickly washed with three changes of KRP, blotted on filter paper, washed once more, blotted again and added in equal portions to the incubating flasks. By this treatment the elimination of any preformed milk in the gland was ensured. Guinea pig glands normally gave sufficient material for four to five flasks.

On a few occasions experiments were performed using mammary gland extracts. These were prepared by homogenizing glands in ice-cold KRP in a tissue homogenizer (Folley & Watson, 1948), centrifuging the suspensions obtained and filtering the supernatants through sintered glass filters.

Incubations. These were carried out in loosely stoppered 150 ml. conical flasks, in KRP buffered at pH 7·4, with air as the gaseous phase. The flasks were shaken for 4 hr. in a water bath at 37°. Substrates were initially present in a concentration of 0·5% (w/v), and the tissues, on a dry-weight basis, in a concentration of roughly 3% (w/v). The volumes were usually 20 ml. for the experiments with cow glands and 15 ml. for those with guinea pig tissue. Control experiments with tissue alone in the absence of substrates were included in all experiments.

Pre-estimation procedure. At the end of the incubation period the mixtures were centrifuged and the solutions fermented for 1 hr. at 37° with washed suspensions of either brewer's or baker's yeast. The latter was used when glucose had been the only substrate. The fermentations were needed to clear the solutions of the substrates (glucose, maltose), or to remove any maltose that might be formed by amylolytic breakdown of glycogen; they also served to destroy a part of the lower dextrins which were formed when glycogen was used as a substrate. This last point will be more fully considered under RESULTS. In many of the guinea pig experiments, however, the glycogen mixtures were treated slightly differently in order to ensure a more complete removal of dextrins. An equal volume of ethanol was added to precipitate glycogen, and the solutions were filtered and evaporated to dryness on a water bath; after redissolving the residues in water, salivary amylase was added and the solutions incubated for 1 hr. at 37°; fermentation was then carried out with brewer's yeast as outlined above.

After centrifuging the fermented solutions, the supernatants were prepared for estimation by methods outlined by Malpress & Morrison (1949*a*). With cow tissue, $Zn(OH)_2$ was used as a deproteinizing agent whenever the substrate had been glucose or maltose; in the guinea pig experiments, however, almost all the solutions were precipitated with ethanol, thus giving a greater uniformity to the pre-estimation procedures adopted for solutions from the experiments with glucose, maltose and glycogen.

Dry weight determinations were made by drying the washed residues from the centrifuged mixtures at 100° overnight.

Estimations. An approximation to the lactose content of the fermented and prepared solutions was first obtained by measuring reduction values by the method of Somogyi (1945) on the equivalent of 1 ml. of the original mixture; these results were then compared with those given by the more specific method for lactose of Malpress & Morrison (1949*a*), usually carried out on the equivalent of 4 ml. of the original mixture. Throughout this paper, results are given as mg. of 'lactose' formed per g. dry weight of tissue, during a 1 hr. incubation period.

Chromatographic analysis of the guinea pig incubates was made on samples of the solutions prepared as above, and deionized by pyridine extraction (Malpress & Morrison, 1949*b*). The experimental technique of Partridge (1948) was used, with only minor modifications. Sugar solutions from any one series of experiments were concentrated to the same final volume before applying to the paper. The final concentration of the sugars present thus varied according to the initial substrate and the degree of synthesis obtained; the usual apparent concentrations, judged from the estimation values, ranged from 0·5 to 4·0%, but, as will be seen, the actual lactose concentrations, especially those from the glycogen experiments, were in fact much lower.

It was found that, under our conditions, the R_F value for a given sugar was not always the same from day to day, and it has been our practice to run control solutions of pure sugars on the chromatograms for direct comparison with the sugars in the experimental solutions (cf. Jermyn & Isherwood, 1949).

RESULTS

Cow glands

After several experiments had been performed on these glands with glucose, glycogen and maltose as substrates, it became clear that they did not provide suitable material for studies on lactose precursors. The results obtained, however, are given here for their interest in relation to the contrary findings of Knodt & Petersen (1945).

Glucose experiments. The results of ten experiments showed that after incubating mammary tissue from cow udders with glucose, there was a slight increase in non-fermentable sugar reduction values in all except one instance, where there was no change; on the other hand, there was no evidence of lactose synthesis by the more specific methylamine method, and it was clear that any increase in the reduction values was not due to the formation of this sugar. It was repeatedly shown that amounts of lactose equivalent to those apparently determined by reduction were readily assessed by the methylamine method when added to the fermented and prepared mixtures, and also that small amounts of lactose added to mixtures at the start of an experiment were recovered in nearly theoretical yields. The ranges and mean values of 'lactose' synthesis given by cow tissue in the presence of glucose are given in Table 1.

Glycogen experiments. It was at first thought that lactose was being rapidly synthesized when glycogen was present as substrate. By reduction, seven experiments showed a mean synthesis of 8·5 mg. per g. dry weight of tissue in the unfermented mixtures after precipitation of glycogen, whilst by the methyl-

amine method a corresponding value of 21·0 mg. was obtained; the mean synthesis in all the eleven experiments in which lactose was estimated by the methylamine method was 19·9 mg. The lack of agreement between the figures given by the two methods clearly suggested that a substance, or substances, other than lactose was being estimated by the methylamine method; but the presence of maltose, the only other likely substance known at that time to give the methylamine reaction, could scarcely be held to account for the discrepancy, since for both methods the calibration curves for this sugar are very close to those of lactose. Fermentation with brewer's yeast, however, under conditions in which comparable amounts of maltose added to the mixtures were fermented in 15–20 min., showed that reducing substances, capable of giving the methylamine reaction, and far more slowly fermentable than maltose, were present in these solutions. A typical fermentation experiment gave the results recorded in Table 2. In one such experiment both the reducing power and the methylamine value were negligible after fermentation for 4 hr. with brewer's yeast.

Table 1. *Formation of 'lactose' in solutions from glucose incubates (cow)*

Method	Mean (mg./g. dry wt.)	Range (mg./g. dry wt.)
Reduction	0·4	0 –1·4
Methylamine	–1·0	–2·1–0·2

Table 2. *Evidence of slowly fermentable products in solutions from glycogen incubates (cow)*

Time of fermentation (hr.)	Reducing value (as mg. of 'lactose')	Methylamine value (as mg. of 'lactose')
0	12·0	23·0
1	5·3	12·3
2	4·6	10·0
4	2·0	3·8

Further work on the nature of this fermentable material showed that after hydrolysis with N-hydrochloric acid the reducing power was 2–7 times greater than that of the unhydrolysed solutions; that a similar substance could be prepared by incubating starch with mammary tissue, glycogen with liver tissue, or either of these substrates with salivary amylase when the enzyme action was stopped at the achromic point; and finally that salivary amylase if allowed to act upon a preparation of the slowly fermentable material converted it into a reducing substance, readily fermentable by brewer's yeast, which was apparently maltose.

We concluded from these experiments that the 'lactose' formed in the glycogen experiments was in reality a variable mixture of lower dextrins and maltose, produced by amylolytic action; the dextrins were soluble in 50 % (v/v) ethanol, since this treatment was used in precipitating glycogen from the solutions, and also, were capable of giving a strong methylamine test.

Maltose experiments. No evidence of the formation of lactose from maltose was found, an increase in reduction values being offset by variable negative or slightly positive methylamine figures. The results thus closely resembled those given by glucose.

Guinea pig glands

Glucose incubates. Unlike the cow glands, guinea pig tissue gave marked evidence of the formation of lactose from glucose when the synthesis was judged by the increase in reducing power of the solutions and also by the methylamine colour reaction. As will be noted later, this evidence was borne out by chromatographic analysis. The results of twelve experiments are given in Table 3. Experiments in which fermentation was prolonged for periods up to 4 hr. gave results from which it may be concluded that all fermentable material had been removed from the solutions recorded in Table 3.

Usually the ratio 'lactose by reduction/lactose by methylamine' (R/M), was greater than one, a result which we interpret as indicating the greater specificity of the methylamine method; occasionally, however, the ratio fell below unity indicating that in some cases the methylamine method was not proving wholly specific for lactose in these estimations on glucose mixtures. We may note, however, that the only substances, other than lactose, at present known to give the methylamine reaction are maltose and cellobiose, both disaccharides with a 1:4-glucosidic linkage, and the lower dextrins already commented on above. None of the other common products of glucose metabolism gives the reaction, nor have we found the reaction with other tissues incubated in the presence of glucose. Since, therefore, the non-lactose reactant in these experiments (*a*) clearly

Table 3. *Formation of 'lactose' in solutions from glucose, glycogen and maltose incubates (guinea pig)*

	'Lactose' by reduction (R) (mg./g. dry wt.)		'Lactose' by methylamine (M) (mg./g. dry wt.)		R/M
Substrate	Mean	Range	Mean	Range	Range
Glucose	7·2	1·7–13·0	5·3	1·0–10·0	0·5–2·8
Glycogen	2·9	1·3– 5·1	7·2	3·8–16·1	0·1–1·2
Maltose	0·6	–0·4– 1·6	0·4	–0·6– 1·2	—

derives from glucose (there was no methylamine colour in control mixtures) and (*b*) only appears when lactose is being synthesized (under similar conditions cow glands failed to give any colour at all), we feel justified in associating it closely with the metabolic pathway of lactose synthesis. We also feel justified in suggesting tentatively that the methylamine values give, in fact, a measure of lactose plus its immediate, and probably non-reducing, precursor (possibly a sugar-1-phosphate), and that in any case they afford a more useful indication of the synthetic activity of the gland than do the changes in reducing power.

Glycogen experiments. Four-hour fermentations showed that slowly fermentable dextrins were present after incubation when glycogen was the substrate. They were even less readily fermentable than those formed by cow tissue. Accordingly, prefermentation treatment with salivary amylase was adopted as routine for these solutions in an attempt to remove the dextrins completely (see Experimental section). The results of six experiments on glycogen mixtures given this revised treatment are shown in Table 3. Analysis by paper chromatography gave direct proof of the presence of small amounts of lactose in these clarified solutions, but it is reasonably certain that in most of these the high methylamine values were largely due to the incomplete removal of dextrins by ptyalin action, a theory supported by the low R/M ratios and by chromatographic evidence (see below).

Maltose experiments. No evidence was obtained for the formation of lactose from maltose by the guinea pig mammary gland. The results of three experiments are included in the data given in Table 3.

Synthesis from lactate. A single experiment in which mammary tissue from a guinea pig was incubated with glucose, glucose plus sodium lactate (lactate 0·5% w/v), and sodium lactate alone (lactate 0·5% w/v), showed the inability of the gland to form lactose from lactate, or to utilize this substrate in increasing lactose formation from glucose (Table 4).

Table 4. *Effect of lactate upon 'lactose' synthesis by the guinea pig gland*

Substrate	'Lactose' by reduction (mg./g. dry wt.)	'Lactose' by methylamine (mg./g. dry wt.)
Glucose	13·0	7·3
Glucose + Na lactate	11·3	7·6
Na lactate	0·4	1·2

Use of extracts

No lactose synthesis could be shown by extracts of any gland, cow or guinea pig, whatever the substrate used. This is in accord with the work of Grant (1936).

Chromatography

The formation of lactose when either glucose or glycogen were the substrates for slices of active guinea pig mammary gland was confirmed by the analytical methods of paper chromatography. In four out of five comparisons the lactose-reduction spot from the glucose mixture was much more intense than that from the glycogen experiment from the same gland. The exception showed a greater synthesis from glycogen, but the abnormality seems to have been a very poor lactose yield from glucose, for some unknown reason, rather than an enhanced yield from glycogen.

Comparison between experiments with glycogen and maltose with the same glands showed formation of lactose from glycogen but none from maltose.

Although quite definite, the lactose spots from glycogen experiments were frequently weak, thus bearing out the significance already given to the high methylamine values and low R/M ratios quoted in Table 3. The nature of the residual colour-developing substances in these solutions was made more certain when paper chromatograms of the hydrolysed solutions were prepared. These showed a very intense glucose spot which again strongly suggested the presence of some lower dextrins in the unhydrolysed mixtures.

DISCUSSION

Our results with cow mammary gland tissue are of interest in that they disagree with the findings of Knodt & Petersen (1945); whereas our work leads us to the conclusion that neither glucose, glycogen nor maltose are suitable substrates for lactose synthesis by cow mammary glands, which were apparently lactating and were collected direct from slaughter, Knodt & Petersen (1945) claimed lactose synthesis from all these substrates in the presence of similar tissue, similarly collected.

Critical examination of the method for estimating lactose which these authors used (Scott & West, 1936) suggests that, although it is applicable to simple mixtures of sugars, e.g. glucose, galactose and lactose, for which purpose it was, apparently, originally designed, it must be used with only the greatest caution in any work involving biological systems of greater complexity, where contaminants may be present which can give rise to reducing substances on hydrolysis. The best illustration of the pitfalls of this method when applied to the problem of lactose synthesis by the mammary gland arises out of our demonstration of the presence of amylolytic activity in all the glands, cow or guinea pig, with which we have dealt. It would be irrelevant at this point to discuss whether or no this activity arises from a true mammary amylase, or is due to enzymes from blood or tissue fluids adsorbed on the

tissue; but at least the activity persists after extensive washing of the tissue slices, and the fact remains that dextrins, and probably maltose too, are formed in these incubations when glycogen is the substrate; these substances, by giving rise to glucose on hydrolysis would lead to completely false estimates of lactose synthesis from glycogen by the Scott & West (1936) method.

Knodt & Petersen's (1945) further claim that glucose and maltose also promote lactose synthesis in the cow gland cannot be explained in identical terms, for it is unlikely that in these cases there could have been any appreciable formation of dextrins in their incubated solutions; but an instance of the inherent dangers of the Scott & West (1936) method in these experiments, even when simple sugars are used as substrates, is given by some experiments we ourselves carried out on cow glands with glucose as the substrate, using the Scott & West (1936) method for estimating lactose. Contrasting with the small increase in reducing power given by our normal, direct reading of the solution after fermentation (mean value as 'lactose' 0·4 mg.), and the slightly negative methylamine values, we obtained an apparent lactose synthesis by the Scott & West method (modified to the extent of using N-hydrochloric instead of N-sulphuric acid for the hydrolysis, neutralizing with N-sodium hydroxide and estimating reducing power by Somogyi's (1945) method) of 6·2 and 5·5 mg. per g. dry weight of tissue in a 1 hr. incubation period. Knodt & Petersen's (1945) corresponding mean value is 2·2 (13·3 for a 6 hr. incubation period), a figure obtained by multiplying their wet weight value by 6·5. This factor for the conversion of wet to dry weights was derived from our own measurements on cow mammary tissue, and is presumably applicable here with only small error. Although it is unlikely that dextrins were responsible for these high readings, it is possible to suggest other substances, of which glucose-1-phosphate is the most obvious, which could interfere with the Scott & West method in very much the same, misleading, way.

While, therefore, the evidence from arterio-venous sampling *in vivo* abundantly suggests that glucose is a precursor of lactose in the cow udder, we feel that the synthesis of lactose by cow tissue has not yet been satisfactorily demonstrated *in vitro*. Since in this work, and in that of Grant (1935), there has been no difficulty in proving synthesis of lactose *in vitro* by the guinea pig gland, it is pertinent to inquire why cow glands should prove to be so refractory. Three suggestions may be put forward at this time. First, we may suppose that the glands used, although milky in appearance were, in fact, regressive and had lost the power of synthetic activity; this supposition remains untested. Secondly, it could be claimed that since no special precautions were taken when collecting the udders from the slaughterhouse, the tissues may have undergone some permanent damage in the 1·0–1·5 hr. between slaughter and the start of the experiment, sufficient to prevent lactose synthesis. We have attempted to check this possibility by comparing the lactose synthesis from glucose given by slices of the same guinea pig gland, cut and incubated (*a*) immediately after killing, and (*b*) after the gland had remained at room temperature for 1·5 hr., just moistened with a few drops of Krebs Ringer phosphate solution. The results showed no appreciable change in the ability of the gland to form lactose, the synthesis by reduction being (*a*) 6·5 mg., (*b*) 4·6 mg. and by the methylamine method (*a*) 4·5 mg., (*b*) 4·3 mg. We might, therefore, tentatively exclude this possibility of a deterioration of active udder tissue in the interim period between killing and the start of an experiment. There remains the third possibility that glucose alone is insufficient as a precursor for lactose synthesis by the cow gland, and that this gland differs from that of the guinea pig in requiring an additional substrate or substrates. Graham, Jones & Kay (1936), and others, have drawn attention to the very high rate of blood flow through the mammary gland which would be required to satisfy the needs for lactose synthesis if blood glucose were the only precursor in the cow, and the suggestion derives further support from the recent work of Folley & French (1948) on comparative differences in the fat metabolism of the mammary glands of ruminants and small laboratory mammals. Their demonstration of the ability of ruminants to utilize acetate in fat synthesis, and their later observation that the trivial acetate uptake by the rabbit mammary gland could be greatly increased by simultaneous glucose administration (Folley & French, 1949) are results which might well have a counterpart in the field of comparative carbohydrate metabolism.

Our results with guinea pig glands agree with those of Grant (1935) in establishing glucose as a precursor for lactose in this species, and extend his observations to include glycogen as a second source of the disaccharide. The fact that glucose customarily gave the larger lactose synthesis makes it less likely that glycogen is an obligatory intermediary in lactose formation (Knodt & Petersen, 1946), and implies rather that its role is one of a minor carbohydrate reserve. Further, since maltose gives rise to no lactose when used as the substrate for mammary tissue, it is most improbable that the metabolic pathway from glycogen to lactose will involve any amylolytic breakdown; we may therefore postulate at this stage the likelihood of a phosphorolytic scheme of glycogenolysis in which either glucose-1-phosphate or glucose-6-phosphate may prove to be a common intermediary on both the glycogen and glucose pathways to lactose formation.

SUMMARY

1. The synthesis of lactose from glucose and glycogen by tissue slices of guinea pig mammary gland has been demonstrated chromatographically, and an attempt to evaluate the synthesis quantitatively has been made by a consideration of the results of two entirely unrelated chemical estimations.

2. No lactose synthesis from glucose or glycogen by tissue slices of cow mammary gland could be demonstrated; a critical assessment of previous claims leads to the conclusion that no *in vitro* demonstration of lactose synthesis by the ruminant gland has yet been made.

3. Maltose (cow and guinea pig glands) and lactate (guinea pig gland) proved ineffective as substrates for lactose synthesis.

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The Valences of Protein Ions from Electrophoretic and Membrane Potential Measurements

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The moving boundary method of electrophoresis, developed by Tiselius (1930, 1937), Philpot (1938), Longsworth (1939), and Svensson (1939, 1940), has many applications, including the small-scale preparative separation of protein mixtures. The purpose of the present studies was ultimately to obtain information likely to be of use in the electrophoretic separation of interesting fractions from pathological human sera.

Convection, due to various causes, is a major difficulty in electrophoresis. The passage of a boundary through the bottom channel of the U-tube due to any cause sets up gravitationally unstable conditions and hence convection, and this is true of the so-called δ-boundary, the origin of which has been discussed by Longsworth & MacInnes (1940).

The separation of pure samples of the components *A* and *B* of a binary protein mixture is illustrated in Fig. 1. If diffusion is neglected, the best possible separation is shown in Fig. 1*a*. If the δ-boundary is eliminated, or reduced to such an extent that its passage through the bottom channel of the U-tube results in negligible convections, the separation theoretically possible is improved to that shown in Fig. 1*b* (Svensson, 1948).

The practical elimination of the δ-boundary may be accomplished either by dilution of the dialysed protein solution (Longsworth & MacInnes, 1940), or by the use of a supernatant buffer solution rather more concentrated than that used for the dialysis (Wiedemann, 1947). There are some objections to these procedures (Pedersen, 1948). Thus, for analy-