to be located in the cell membrane, the region believed to be the site of many mitochondrial functions. It would be rash to ascribe to the quinones a function on electron-transport on these grounds alone, for in *M. lysodeikticus* over 90% of the total lipid of the organism is found in the membrane (Macfarlane, 1961). This is the region, therefore, in which lipid components would be expected to occur. The present findings are, however, quite consistent with a respiratory function for these quinones.

SUMMARY

1. The intracellular distribution of ubiquinone and vitamin K in certain bacteria has been investigated.

2. Suspensions of *Escherichia coli* were frozen and the cells broken by mechanical shock in the Hughes (1951) bacterial press. Both the vitamin K and the ubiquinone of this organism were found exclusively in the insoluble cell residues, representing the cell walls plus the plasma membranes.

3. Protoplast membranes were obtained from *Micrococcus lysodeikticus* by treating the cells with lysozyme followed by differential centrifuging. The entire vitamin K content of the organism was recovered from the membranes. This organism does not contain ubiquinone.

4. *E. coli* and *M. lysodeikticus* were subjected to progressive ultrasonic disintegration. The rate of liberation of ubiquinone and vitamin K in *E. coli*,

and of vitamin K in *M. lysodeikticus*, indicated that these quinones were components of the cell 'hulls', i.e. the cell walls plus attached cell membranes.

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The Onset of Lactose Synthesis after Injection of Prolactin

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The synthesis of lactose in the mammary gland was first demonstrated unequivocally by Grant (1935), who proved lactose synthesis in slices of guinea-pig mammary gland by fermentation with adapted yeast. Malpress & Morrison (1949, 1950) also demonstrated lactose synthesis in mammary glands, by preliminary fermentation with brewer's yeast followed by a non-specific trimethylamine method. Paper-partition chromatography of sugars (Partridge, 1948) was adapted for the quantitative estimation of lactose in the mammary gland by Heyworth & Bacon (1955), who confirmed the

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synthesis of lactose in surviving mammary-tissue slices from lactating guinea pigs. Other ways of estimating lactose, such as the chloramine-T method adopted by Folley & Greenbaum (1947) and the orcinol method described by Slater (1957), lack the specificity given by preliminary chromatographic separation of the sugars. For this reason the chromatographic method was adopted to study the initial stages of lactose synthesis in the mammary glands of the rabbit. In order to measure the concentration of lactose at the first possible moment after its appearance in the mammary gland, possibly before any milk appeared in the alveoli, it was necessary to concen-

trate the supernatant mammary extract before chromatography. This was done by freeze-drying. As this also led to an increase in the salt concentration in the extracts which interfered with the chromatographic separation of the sugars it was first necessary to de-ionize the solutions. A description of the modified method for lactose estimation is followed by an account of the course of lactose synthesis in the mammary glands of intact rabbits after injection with prolactin (lactogenic hormone).

MATERIALS AND METHODS

Young virgin female rabbits of the Dutch and New Zealand White strains, weighing between 2.5 and 4.5 kg., were used. They were made pseudopregnant, to develop the mammary glands so that they would respond to injections of prolactin, by giving a single intravenous injection of 50 i.u. of human chorionic gonadotrophin (Pregnyl, Organon Laboratories Ltd.) 11 days before the mammary-intraductal injections of prolactin (Bradley & Clarke, 1956). There are four pairs of mammary glands in the rabbit, occupying almost the whole of the ventral side of the thorax and abdomen. Each gland is served by a central teat and is divided more or less radially into anatomically separate compartments or 'sectors', the ducts opening separately on the teat. Each sector can respond to lactogenic substances introduced through the galactophore independently of the adjacent sectors (Lyons, 1942). At intervals after the mammary-intraductal injections the rabbits were killed by cervical dislocation. The ventral skin was reflected back to expose the mammary glands and a visual estimate was made of the local lactogenic response in terms of the amount of milk visible.

The injected mammary sectors were dissected out, weighed, chopped finely, homogenized in a top-drive blender (Folley & Watson, 1948) and made up to a measured volume (about 20 ml.) with water. The cell debris was removed by centrifuging if the extracts were to be subdivided at this stage to give replicate lactose estimates. Zinc sulphate and barium hydroxide were added to precipitate the protein (Nelson, 1944) and two 1 ml. portions removed for reducing-sugar estimation by the Somogyi (1945) modification of the Nelson (1944) method. A volume of supernatant estimated to contain about 1 mg. of reducing sugar, up to a maximum volume of 15 ml., was de-ionized by passage through a column of mixed-bed resin (Bio-Deminrolit, The Permutit Co. Ltd.), 6 mm. diam. $\times$ 130 mm. To avoid any loss of sugar on the column it was first necessary to convert the cation-exchanger in the resin into a weak acid salt (Hulme, 1953). Enough resin for about 50 runs was treated in a column with 25 bed-volumes of 2N-acetic acid followed by thorough washing with 60 bed-volumes of water. The treated resin was stored in water and each column was washed through with 30 ml. of water before use. The de-ionized solutions were freeze-dried in a vacuum desiccator, with silica gel as the desiccant. To avoid any cumulative error arising in successive applications of the dilute sugar solution to a spot on the chromatogram, an internal standard of maltose was used. An Agla micrometer syringe (Burroughs Wellcome and Co. Ltd.) was employed to add 0.2 ml. of aqueous 0.45% maltose

solution to each evaporating basin containing the freeze-dried material. The contents of each basin were then divided more or less equally between two adjacent spots on a chromatogram with capillary tubing and miniature rubber teats (British Drug Houses Ltd.). The spots were dried between each application with a hair-drier (cold air). Six spots were arranged equidistantly across one end of a sheet (23 cm. $\times$ 50 cm.) of Whatman no. 1 paper. Preliminary washing decreased the paper blank in the subsequent sugar estimations. A shallow Perspex tank with a false bottom of perforated zinc was used. It was designed to take about 20 sheets of paper of the requisite size at a time. The papers were laid flat and 2 l. of 2N-acetic acid was allowed to filter through, followed by 2 l. of water and then 2 l. of 10N-ammonia solution. Finally the papers were washed with 10 l. of water and hung up to dry. The papers were not touched by hand after this procedure. They were trimmed at one end to six points to allow the solvent, 2,4,6-collidine saturated with water, to drip off evenly as it descended.

After a preliminary equilibration period of 2 hr. at 28° in the atmosphere of collidine-saturated water, in a glass tank, the solvent was added to the trough through a hole in the lid of the tank and development allowed to continue for 56–60 hr. After being dried in a fume-cupboard for 36 hr. the chromatograms were cut into six strips. One from each pair was sprayed with benzidine-trichloroacetic acid (Bacon & Edelman, 1951) and used as a guide to show the positions of the sugar spots. The areas containing the sugars on the other strips were cut out and eluted in 5 or 3.5 ml. of water (Heyworth & Bacon, 1955). Blank areas behind the lactose were also eluted to act as controls. The reducing values of duplicate 1 ml. samples of the eluates were estimated by the Nelson-Somogyi method. Sugar standards were prepared afresh for each estimation. Standards, eluates and reagent blanks were heated in a boiling-water bath for 20 min. and after the addition of colour reagent were made up to 15 ml. Their absorption was measured relative to water at 660 m μ with a Unicam SP. 600 spectrophotometer. The standard curve was drawn for each set of determinations as the slope varied slightly. After the calculation of the amounts of lactose, glucose and maltose present in the spots on the chromatogram, the amount of the internal standard maltose present, relative to the known amount added initially, was used to calculate the amounts of lactose and glucose present in the supernatant fluid, which had been de-ionized, from the mammary gland. This allowed the calculation of the amounts of lactose and glucose present in the whole mammary-gland homogenate.

RESULTS AND DISCUSSION

Recovery of reducing sugars after chromatography. Known amounts of lactose (50 and 500 μ g.), glucose (100 μ g.) and maltose (400 μ g.) were placed in separate spots on chromatograms and eluted after development as described above. The recovery of the lactose was $95 \pm 5\%$ (S.E.M.; three experiments) and $100 \pm 2\%$, the glucose $98 \pm 3\%$ and the maltose $100 \pm 1\%$.

The overall recovery of lactose added to raw mammary-gland homogenates was estimated as

follows. A single homogenate was prepared from all the mammary glands of a pseudopregnant rabbit which had been injected intravenously with 150 i.u. of prolactin (12 i.u./mg.). A little milk secretion was visible in the glands at necropsy. The homogenate was divided into 18 equal parts, i.e. three groups of six replicates. The first group was used to get an estimate of the naturally occurring lactose and glucose. To each part of the second group was added 200 μ g. of lactose and to each of the third group 800 μ g. of lactose. The lactose and glucose content of each homogenate was determined and the results are summarized in Table 1. In this experiment the mean recovery of added lactose was 100%. In four other experiments a total of 32 separate estimates of added lactose, in which the amount added varied between 125 and 1000 μ g., gave a mean recovery of $99.1 \pm 3.3\%$ (s.e.m.). Experiments in which glucose was added to the mammary-gland homogenates gave corresponding results.

Estimates of the reducing sugar in crude mammary-gland homogenates. When the true amount of glucose and lactose present in a particular mammary-gland homogenate was determined, the total was always insufficient to account for the initial

reducing value of the homogenate. The discrepancy ranged from 5 to 28% (Table 2, column 6) and was highest in the homogenates which contained little milk. Table 2 also shows (column 7) that the lactose was only a small proportion of the total reducing sugar in the homogenates which contained little milk, as these had relatively large amounts of glucose.

Course of lactose synthesis in the mammary gland after treatment with prolactin. The first instant at which lactose could be detected in the mammary gland after mammary-intraductal injections of prolactin was investigated. The different strains varied in this respect but in New Zealand Whites, which responded earliest to the intraductal injections, lactose first appeared 3 days after the injections. No lactose was detectable 48 hr. after the intraductal injections. The course followed from the third to the fifth days inclusive in two rabbits is shown in Fig. 1, and the graph is extended towards the origin on the basis of numerous other experiments. This particular experiment was designed primarily for a purpose connected with a possible prolactin assay. Nevertheless, it throws some light on the rate of synthesis of lactose in mammary tissue under the influence of local injections of prolactin. Both rabbits were given four separate mammary-intraductal injections containing different amounts of prolactin, daily, on each of 3 successive days. The rabbits were killed 2 days after the last set of injections and thus the influence of the four different dose levels could be determined at three different time-intervals. The total prolactin injected into each rabbit was 1125 μ g. (potency of prolactin: 15 i.u./mg.). For Fig. 1 the total lactose in the two rabbits for each time-interval for a given total amount of prolactin injected was calculated. This was preferred to the

Table 1. *Replicate sugar determinations and the recovery of added lactose*

Determinations were carried out on six replicate mammary-gland homogenates in each case; the results are given as means $\pm$ s.e.m.

Lactose present (μ g.)	Glucose present (μ g.)	Lactose added (μ g.)	Lactose recovery (%)
149 $\pm$ 1.9	454 $\pm$ 11.0	0	—
353 $\pm$ 7.5	479 $\pm$ 5.7	200	102
940 $\pm$ 12.5	455 $\pm$ 12.0	800	99

Table 2. *Reducing-sugar estimations done on mammary-gland homogenates from a rabbit given mammary-intraductal injections at ten separate sites*

The potency of prolactin A was 12 i.u./mg., and that of prolactin B was 24 i.u./mg. Each mammary-gland homogenate was divided into three parts which were worked up separately. The mean of the three replicates in each case had the following s.e. values: reducing sugar, $\pm 2\%$ in crude homogenate; lactose, $\pm 6\%$; glucose, $\pm 6\%$.

Prolactin injected	Dose (μ g.)	Total sugar present in two replicate mammary sectors (μ g.)			$\frac{B+C}{A} \times 100^\dagger$ (%)	$\frac{B}{A} \times 100$ (%)
		'Lactose' in crude homogenate* (A)	True lactose (B)	True glucose (C)		
A	40	2 136	396	570	72	19
A	160	11 299	7828	895	85	69
B	20	2 832	362	894	76	13
B	80	9 730	7780	545	91	80
B	320	10 254	8157	808	95	80

* Reducing sugar estimated against lactose standards.

† For the purposes of this calculation the molar reducing value of glucose is assumed to be twice that of lactose

lactose per unit weight of tissue as at the higher response levels the milk secretion forms a large part of the weight of the sector and it is not possible to separate the milk from the tissue completely. There was a steep rise in the synthesis of lactose on the fifth day after injection, whereas that of glucose remained relatively constant.

The method described by Heyworth & Bacon (1955) for the study of lactose metabolism in lactating-guinea-pig mammary-gland slices provided a sound basis for studying the initial stages of lactogenesis in the rabbit after intraductal injections of prolactin into the mammary glands. The modifications necessitated by the small amount of milk present at the early stages have made it possible to measure accurately lactose concentrations of about 10 μ g./100 g. wet wt. of tissue.

The discrepancy between the true amount of glucose plus lactose present in the mammary gland and the amount indicated by measuring the reducing value of a crude protein-free homogenate emphasizes the desirability of separating the sugars if the lactose concentration is to be used as a measure of milk secretion or of retained fluid in the mammary gland. The cause of the discrepancy is not clear but may be partly due to the presence of other reducing substances in the mammary-gland suspensions. Heyworth & Bacon (1957), Trucco & Caputto (1954) and Carabelli, Caputto & Trucco (1960) have observed the acidic-reducing substance, neuramin-lactose, in the mammary gland, and this substance would be removed on the ion-exchange column in my experiments. Also, two separate sugar spots were frequently noticed in front of the glucose in the chromatograms of rabbit-mammary-gland homogenates (R_{glc} 1.16 and R_{glc} 1.27). A similar feature has been noted in

rat-mammary-gland extracts (Trucco & Caputto, 1954) and in rat milk (Iwamura, Hidaka & Nonaka, 1956). The occurrence of these substances in the rabbit-mammary-gland extracts was so variable that it was not possible to derive a satisfactory correction factor for the reducing value of the crude mammary-gland homogenate that would relate it accurately to the true lactose content.

No lactose has been detected in mammary glands from untreated pseudopregnant rabbits, or up to 48 hr. after local treatment with prolactin. Kuretani (1957), using a chromatographic method, could not find any lactose in mammary-gland extracts prepared from rats in late pregnancy. On the other hand, Slater (1961) gave figures for the lactose present in late-pregnancy-rat-mammary-gland suspensions of 160 to 580 mg./100 g. of tissue, depending on the method of lactose estimation used. However, he suggested that the methods he employed may not measure lactose primarily in this physiological state of the animal.

The rapid increase in lactose synthesis between the fourth and fifth days after mammary-intraductal injections of prolactin suggests that in this strain of rabbit the relative potency of different preparations of prolactin can best be detected at this stage. If the lactogenic response is to be used for assay purposes the true lactose content gives the best parallel-line assay (see Table 2).

The reducing value of the crude mammary-gland homogenates is nearer to a measure of the lactose content at the higher dose levels as there is more milk present, but it is swamped by the glucose content of mammary tissues at the lower dose levels. If it is desired to measure the response to low dose levels, or in the early stages of lactogenesis, or after mammary-intraductal injections of prolactin in mid and late pregnancy, it is clearly preferable to know the true lactose content.

SUMMARY

1. The initial stages of lactose synthesis in the intact rabbit at the sites of mammary-intraductal injections of prolactin (lactogenic hormone) were studied by a modified chromatographic method that allows the determination of lactose in mammary glands down to a concentration of 10 μ g./100 mg. of tissue.

2. The following features of the lactogenic response are described: (a) the total absence of lactose from non-lactating mammary glands; (b) the sharp rise in lactose synthesis occurring in the mammary glands on the fifth day after local treatment with prolactin; (c) the relationship of the true amounts of lactose and glucose present to the reducing value of a crude mammary-gland homogenate.

Fig. 1. Lactose (●) and glucose (○) present in injected sectors of rabbit mammary glands at various times after mammary-intraductal injections of prolactin.

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The Physiological Role of Cerebron Sulphuric Acid (Sulphatide) in the Brain

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Sulphur is found in mammalian-brain lipid as the sulphate ester of galactocerebrosides (sulphatides) with cerebronic acid, lignoceric acid or possibly neuronc acid as the fatty acid constituent of the cerebroside (Jatzkewitz, 1960). The sulpholipid has been isolated both as the crystalline form sulphatide A and in association with phospholipid as sulphatide B (Lees, Folch, Sloane-Stanley & Carr, 1959). It has been claimed that brain sulphatides continue to accumulate throughout the

life-span of the rat (Koch & Koch, 1913; Green & Robinson, 1960; Bakke & Cornatzer, 1961; Goldberg, 1961). The continual accretion of sulphatide in the human brain has also been reported (MacArthur & Doisy, 1919; Bakke & Cornatzer, 1961). In conformity with these findings, radioactive sulphate is found to be incorporated in relatively small amounts *in vitro* into the lipids of adult-rat-brain slices (Heald & Robinson, 1961), and after injection of [³⁵S]sulphate into rats or rabbits some labelled sulpholipid can be isolated from the brain (Dziewiatkowski, 1953; Holmgard, 1955; Radin, Martin & Brown, 1957; Green & Robinson, 1960; Bakke & Cornatzer, 1961). It was concluded that this radioactive sulphatide was metabolically inert, for no radioactivity was lost from the brain up to 16 days after injection (Radin *et al.* 1957). These results imply that there is a small but continual accumulation of sulpholipid in the brain and that once this lipid is laid down it is retained possibly for the life-span of the animal (Radin *et al.* 1957; Heald & Robinson, 1961).

Previous work (Davison, Morgan, Wajda & Wright, 1959; Davison & Dobbing, 1960) has suggested that the relative metabolic stability of brain lipids is related to their structural role in the