products resulting from the incubation of griseofulvin with *Mucor ramannianus* yielded no recognizable material, although an unidentified phenolic substance, m.p. 142–145°, was isolated from the culture filtrate. From the evidence available, this phenolic substance does not appear to be one of the metabolic products discussed here.

SUMMARY

1. Incubation of griseofulvin with fungi gave rise to various demethylated metabolites. Thus, 7-chloro-4-hydroxy-6:2'-dimethoxy-6'-methylgris-2'-en-3:4'-dione (4-demethylgriseofulvin) was produced by *Microsporum canis*, griseofulvic acid (2'-demethylgriseofulvin) by *Botrytis allii* and 7-

chloro-6-hydroxy-4:2'-dimethoxy-6'-methylgris-2'-en-3:4'-dione (6-demethylgriseofulvin) by *Cerco-sporea melonis*.

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The Effect of Glucose and Acetate on Milk Secretion by the Perfused Goat Udder

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Acetate is now generally accepted as the major energy source for most ruminant tissues. A great deal of evidence, summarized by Folley (1956), suggests that acetate is more important than glucose in the metabolism of the ruminant mammary glands. Not only is acetate directly incorporated into milk fat, but the oxygen consumption of ruminant mammary slices taken during lactation is higher with acetate as substrate than with glucose (Folley & French, 1950). However, recent work in conscious lactating goats (Linzell, 1960) shows that the udder removes on average 10.3 g. of glucose, 3.2 g. of acetic acid, and at least 650 mg. of α -amino acid nitrogen from the blood passing through it for every 100 ml. of milk produced. Compared with the uptake in non-lactating glands, these figures represent a threefold increase in the rate of uptake for acetate but a tenfold increase for glucose. Of the glucose taken up by the lactating gland only 50% can be accounted for as lactose. These considerations suggest an important metabolic function for glucose, apart from its confirmed role in the formation of lactose.

We therefore felt that the influence of acetate and glucose upon mammary metabolism should be re-examined. It has not been possible to do this satisfactorily either in the whole animal or with

tissue slices. In the whole animal, glucose or acetate cannot be eliminated entirely, and, in the tissue slice, it is not easy to measure the amount and composition of milk secreted. These are both possible in an isolated perfused mammary gland and we have used the perfused goat's udder for this purpose. In our experiments the glands are removed under spinal anaesthesia with an anoxic period of only a few minutes and perfused with a simplified perfusate of known composition. With this preparation we have shown that for the first 2 hr. milk is formed from endogenous substrates, but after that it is only produced if a substrate mixture is run into the perfusate continuously at a rate appropriate to the amount of milk being formed. The mixture used contains glucose, acetate, amino acids and minerals in the proportions that are removed from the blood by the lactating goat's udder *in vivo*. In this paper we report the effects of varying the proportion of acetate and glucose in this mixture, leaving the other components constant.

METHODS

The method of perfusion (Hardwick & Linzell, 1960) included the modifications mentioned to maintain a high blood flow throughout perfusion and the incorporation of

an artificial kidney. The glands were removed aseptically under spinal (epidural) anaesthesia. The perfusate consisted of washed goat red cells suspended in a modified Krebs bicarbonate solution [composition (g./l.): NaCl, 7.70; NaHCO_3 , 2.10; KCl, 0.230; CaCl_2 (anhydrous) 0.282; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.294; KH_2PO_4 , 0.162] containing instead of albumin the total proteins of defibrinogenated plasma (70 g./l.). The substrates required for milk secretion were run into the perfusate continuously throughout the perfusion by a motor-driven syringe.

The protein for the perfusate was prepared by adding ether (11% of the plasma volume) to plasma cooled to 0° (Kekwick & Mackay, 1954). The precipitate was removed, and the supernatant dialysed for three 24-hr. periods against 10 vol. of NaCl solution (2–3 g./l.) and concentrated by freeze-drying. The NaCl content was reduced by further dialysis against distilled water and measured by flame photometry before adding the necessary Krebs salts.

The artificial kidney was inserted between a reservoir and the subsidiary circulating pump, and consisted of 10 m. of dialysis tubing 6 mm. in diameter (Visking $\frac{3}{32}$ in.) in 20 l. of Krebs solution to which were added (l.): glucose, 0.5 g.; acetic acid (neutralized), 0.05 g.; L-asparagine, 3 mg.; L-glutamine, 50 mg.; L-cysteine, 35 mg.; L-phenylalanine, 25 mg.; L-tryptophan, 20 mg.; L-tyrosine, 17 mg.; streptomycin and penicillin, 100 000 units; casein hydrolysate to give a total α -amino N value of 50 mg./l. Glucose or acetate was omitted from the 'kidney' when not given in the substrate solution.

The casein hydrolysate was made by refluxing 100 g. of casein in 25 ml. of formic acid (90%; density 1.2), 60 ml. of conc. HCl and 500 ml. of water for 3 days. As much acid as possible was removed by evaporating the resulting material under reduced pressure to a small volume, adding 200–300 ml. of water and re-evaporating. This evaporation was repeated three times. Activated carbon (20 g.) (Norite NK; Hopkin and Williams Ltd.) was added, the solution filtered and neutralized, and the α -amino N content checked.

The substrate solution used consisted of the mixture of amino acids and phosphate used by Hardwick & Linzell (1960). The total concentration of glucose plus acetic acid (neutralized with NaOH) was 100 g./l., but the proportions were varied in different experiments. This substrate solution was run into the perfusate continuously at a rate determined hourly by the milk yield of the previous hour. A solution of CaCl_2 (0.23 g./100 ml.) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (67 mg./100 ml.) in water was also added at a rate of 8 ml./hr.

Milk and perfusate were analysed as described by Hardwick & Linzell (1960). Arterio-venous differences of O_2 and CO_2 were measured by taking arterial and venous samples simultaneously and analysing for O_2 and CO_2 by the standard manometric procedure of Van Slyke (Peters & Van Slyke, 1932). Consumption of O_2 and R.Q. were calcu-

lated from these figures and the blood flow at the time. Ketone bodies were measured in some experiments by the methods of Bakker & White (1957) and Werk, McPherson, Hamrick, Myers & Engel (1955). Glucose was sometimes checked with the glucose-oxidase method of Huggett & Nixon (1957).

Milk yields during perfusion were compared with the average hourly yield of the gland on the goat during the previous week and this is referred to as the yield before the experiment. The composition of the total milk secreted for the 3 days before the experiment was used for comparison and is referred to as 'control level'.

RESULTS

Hardwick & Linzell (1960) have shown that the milk yield in the isolated udder is dependent on blood flow and the quantity of substrates provided. In the present experiments blood flow, after the initial phase, was 35–65 ml./100 g./min., which is in the normal range (47 ± 9) for conscious lactating goats. Also, more substrate mixture was given than would have been necessary *in vivo* to make the amount of milk obtained during the perfusion. In addition, nutrients diffused into the perfusate from the artificial kidney when concentrations in the perfusate fell below those in the 'kidney', and vice versa. We therefore consider that neither low blood flow nor restriction of substrates was a factor in these experiments.

Eight glands from four goats were perfused. Since the variation in milk yield between the two glands from one goat is less than that between glands of different goats the basic experimental design shown in Table 1 was used. In addition to comparing the results obtained under these conditions, one with another, it is important to compare the glucose, acetate and amino acid uptake/100 ml. of milk *in vivo* (see introduction) and in the perfused gland, as a measure of efficiency of the latter. Comparison of O_2 uptake and R.Q. *in vivo* and during the perfusion is also of significance (see Fig. 5).

Perfusions with glucose plus acetate

In these two experiments the substrate solution run in and the 'kidney' fluid contained glucose plus acetate (3:1, w/w; 1:1 molar ratio). The results were similar to those reported by Hardwick & Linzell (1960) and are shown in Figs. 1L and 2R. We have previously found that milk secretion during the first 2 hr. is largely from endogenous

Table 1. *Experimental design*

Experimental design showing substrates, other than amino acids, Ca, Mg and phosphate, added continuously from the start of perfusion. Alterations in substrate composition were made subsequently in glands 2L, 3R, 4L and 4R. G, Glucose; A, acetate.

	Goat ...	1	2	3	4
Gland	Left	G + A	A	G	A and little G
	Right	G	G + A	A	No addition

substrates (see also Fig. 4*R*). The average rate of secretion after this was 41 and 47 % of the yield before the experiment and was still above 10 % at the 12th hr. The milk composition was essentially normal. The O₂ uptake was equal to that in conscious goats in declining lactation and the R.Q. was higher (Fig. 5). For each 100 ml. of milk formed the two glands took up 12 g. (estimated by using final perfusate concn. and assuming equilibrium with the 'kidney' fluid) and 19 g. (based on analysis of

Fig. 1. Expt. no. 1. *L*, Left gland: glucose plus acetate; *R*, right gland: glucose only. The perfusion was begun at 0 hr.; points before this represent milk secreted during the removal of the glands under epidural anaesthesia. The average hourly yield for 7 days before the experiment equals 100%. Horizontal broken lines represent composition of milk produced during the 3 days before experiment; for O₂ and the R.Q. the broken lines indicate average values in conscious lactating goats (see Fig. 5).

perfusate and 'kidney' fluid) of glucose, 0.76 and 0.54 g. of α -amino N and 6.8 and 6.6 g. of acetate. The greater uptakes of glucose in one case and N in the other were associated with higher concentrations in the milk of lactose and N respectively.

Perfusion with glucose only

In both these experiments (Figs. 1*R* and 3*L*) the average rate of milk secretion after the 2nd hr. was 30% of the yield before the experiment. The milk

Fig. 2. Expt. no. 2. *L*, Left gland: 0-3 hr., acetate only; 3-11 hr., glucose and acetate; KCN (0.1 mM) was added at 9.3 hr. *R*, Right gland: glucose and acetate. Explanations are as in Fig. 1.

composition was in the normal range and similar to that with glucose and acetate, except that the fat concentration fell after the first 2 hr., eventually to as low as 18 and 12% of the control level. The O_2 consumption was significantly

($P < 0.05$) lower than with acetate and glucose and the R.Q. < 1 ($P < 0.02$) (Fig. 5). For each 100 ml. of milk formed the two glands took up 28 g. (estimated as above) and 23 g. of glucose and 0.98 and 0.66 g. of α -amino N. Again the higher

Fig. 3. Expt. no. 3. L, Left gland, glucose only; R, right gland, 0-4 hr. acetate only; 4-12 hr. glucose + acetate. Explanations are as in Fig. 1.

uptake of N corresponds with a higher total milk N.

Perfusion with acetate only

In these two experiments (Figs. 2*L* and 3*R*) the milk yield fell by the 3rd hr. to a very low level and

was not significantly different from glands receiving no substrates (e.g. Fig. 4*R*). The samples of milk that were large enough to analyse showed a fall in lactose and a rise in casein and fat concentration. The O_2 uptake was similar to the value with glucose only and intermediate between those

Fig. 4. Expt. no. 4. *L*, Left gland, 0-3 hr. The substrate solution contained glucose + acetate (1:27 molar ratio); 3-5 hr. (1:5 molar ratio); 5-10 hr. (1:1 molar ratio). Arrows indicate where glucose in substrate solution was increased. *R*, Right gland: 0-4 hr., no substrates; 4-6 hr., acetate + fumarate (18:1 molar ratio); 6-12 hr., glucose + acetate (1:1 molar ratio). Other explanations are as in Fig. 1.

of starved glands and those receiving glucose plus acetate. The R.Q. was markedly raised (Fig. 5). Because of the very low milk yields it seemed desirable to test the glands' ability to secrete milk. At 3 hr. in one experiment and at 4 hr. in the other, therefore, we added glucose to the 'kidney' fluid and to the perfusate to give a concentration of 50 mg./100 ml., and changed the substrate mixture being infused from one containing acetate to one containing glucose and acetate (molar ratio 1:1). There was an increase in milk yield at the next hour and thereafter it was similar to the controls receiving glucose and acetate throughout (Figs. 2*L* and 3*R*). The composition of the milk obtained after the addition of glucose showed a rise in the concentration of lactose to that seen in experiments where glucose was given throughout. In addition, initially, there was a very marked rise in the fat and casein concentration, which fell again to more normal levels as the perfusion progressed.

Acetate plus a little glucose

Since so little milk was produced with acetate alone an experiment was carried out (Fig. 4*L*) to see whether milk secretion was possible with a substrate solution and 'kidney' fluid containing a smaller ratio of glucose to acetate than 3:1 (w/w) (1:1 molar ratio). One gland given glucose and acetate (1:9, w/w) (1:27 molar ratio) for 3 hr. produced no more milk than the opposite gland getting no substrates. When the ratio in the substrate solution and 'kidney' fluid was increased to 3:5 (w/w) (1:5 molar ratio) for 2 hr. there was only a slight increase in yield. To test the ability of the preparation to secrete, we added glucose to the perfusate and 'kidney' fluid (50 mg./100 ml.) at 5 hr. and changed the substrate solution to glucose, amino acids and phosphate only, because at that

time the acetate level of the perfusate was high. The milk yield increased during the subsequent 4 hr. to 44 % of the yield before the experiment. The level of acetate in the perfusate fell to normal within 2 hr. so that we had to add it in the substrate mixture again.

Acetate plus fumarate

Turner (1956) has shown that fumarate stimulates fatty acid synthesis by mammary-gland slices in the presence of acetate. We therefore tested acetate plus fumarate as substrates on the gland (Fig. 4*R*) that was starved for the first 4 hr. At the end of the 4th hr. fumarate (5 mM) and acetate (1 mM) were added to the perfusate and the 'kidney' fluid, and the infusion of the basic substrate mixture plus acetate and fumarate (9:1, w/w; 18:1 molar ratio) was begun. There was no stimulation of milk secretion during the next 2 hr. At the end of the 6th hr. we added glucose (50 mg./100 ml.) to the perfusate and 'kidney' fluid (which still contained acetate and probably fumarate), and changed to a substrate solution containing (in addition to amino acids and phosphate which were always present) glucose, instead of acetate and fumarate. There was an increase in milk secretion during the subsequent 4 hr. (see Fig. 4*R*).

Formation of ketone bodies

Kronfeld & Kleiber (1959) have recently suggested that the udder itself is a source of ketone bodies in bovine ketosis, and we have looked for this in Expts. 3 and 4 by analysing the mammary lymph for total ketone bodies. All the values were below 15 mg. of acetone/100 ml. and there was no significant difference in the concentrations whether the glands were getting glucose or not.

DISCUSSION

Our results confirm the importance of acetate in the formation of milk fat, and of glucose in the formation of lactose. In addition, glucose is shown to be essential for milk secretion in our experiments.

Role of acetate. By the injection of labelled acetate into lactating goats, Popják, French, Hunter & Martin (1951) confirmed that acetate is the major source of milk fatty acids up to and including C_{18} . These acids form about 75 % of milk fatty acids by weight in the goat. Our results are consistent with their findings because, in the absence of acetate, the milk fat fell to less than 20 % of its previous value. This level of 20 % corresponds roughly with the higher (C_{18} and above) fatty acid content of normal milk. In the whole animal, apart from acetate, the glands take up enough fatty acids (free and esterified) from the

Fig. 5. Oxygen consumption and R.Q. (mean $\pm$ S.E.M.) of perfused udders with different substrates. The horizontal broken lines represent average values for conditions *in vivo* (conscious lactating goats) taken from Linzell (1960). O₂: upper line, goats in full lactation; lower line, in declining lactation. R.Q.: average value at all stages of lactation.

plasma to account for a major proportion of total milk fat. In our experiments only acetate was given so that the metabolism may have been abnormal. Nevertheless, fat in variable amounts was detected histologically in the secretory cells at the end of the experiment, and tests in previous perfusions have shown that neutral fat in the presence of acetate (Hardwick & Linzell, 1960) did not increase the milk yield or milk fat content, suggesting that the perfused tissue may have large stores of endogenous fat. However, the preparations then used were less active, and such experiments should be repeated under present conditions.

Role of glucose. The dependence of lactose synthesis on glucose was first demonstrated by Foa (1912) in the perfused sheep udder. He showed that lactose could be detected in the milk when glucose was present in the perfusate, but not when it was absent; galactose in the perfusate appeared unchanged in the milk. Kleiber *et al.* (1955) showed that 80% of milk lactose in the intact cow came from the 'plasma glucose pool'. In the conscious goat, 50% of the glucose taken up by the udder can be accounted for as lactose. We expected, therefore, that leaving out glucose from our perfusate would decrease the milk lactose, just as leaving out acetate decreased the fat concentration: this did occur. More striking than this fall, however, was the profound fall in the total milk obtained, resembling that observed when no substrates were given.

Some possible causes of the lowered rate of milk production have been eliminated. It is possible that the milk was secreted (i.e. passed into the alveolar lumina) but that the absence of glucose or the high concentrations of acetate interfered with milk ejection (i.e. prevented oxytocin from acting on the myoepithelial cells), thus allowing milk so secreted to accumulate. This is unlikely because the milk yield after glucose had been added was not greater in volume than that obtained at a similar time in experiments with glucose plus acetate given throughout, indicating that, in the absence of glucose, secretion of fluid had not been occurring. The possibility that secretion was inhibited, in the present experiments without glucose, by the high level of acetate reached in the perfusate is eliminated, since secretion started as soon as glucose was added, while the acetate level was still high. Therefore our present evidence clearly suggests that in the complete absence of glucose the secretion of milk fluid ceases, even though adequate quantities of acetate and amino acids are available.

In the absence of glucose the concentration of fat and protein rose in the small amounts of milk formed. This has also been seen in two out of six glands starved completely (Fig. 4*R*), and in one

gland given adequate quantities of amino acids but very low amounts of glucose and acetate (Hardwick & Linzell, 1960; Fig. 6). The rise in the concentrations of fat and protein at the time when the lactose concentration was falling suggests that protein and fat synthesis continue so long as the specific precursors are available (they were in the present experiments and will be to a variable degree in starved glands depending on the level of endogenous substrates). If fluid secretion and lactose formation fail because there is no glucose then fat and protein, having precursors available, might be expected to accumulate in the cells until a critical concentration is reached. In this case once fluid secretion is re-established the first milk formed should have a raised concentration of fat and protein, and in fact this occurred.

Since acetate was present in high concentration and there was an oxygen uptake in excess of that in the absence of all substrates, it may be assumed that the Krebs cycle was functioning in the absence of glucose. This belief is strengthened by the failure of fumarate in the absence of glucose to have any effect on milk secretion or O_2 uptake (cf. Turner, 1956). Therefore it seems that the energy for secretion comes from some pathway of glucose metabolism other than the Krebs cycle and one which is not available to acetate.

One such metabolic route is the pentose phosphate pathway. Recent evidence from both ruminant (Black, Kleiber, Butterworth, Brubacher & Kaneko, 1957) and non-ruminant (McLean, 1958) mammary tissue indicates that this pathway is an important route of glucose degradation in lactating but not in non-lactating mammary tissue. McLean (1960) has suggested that the pentose phosphate pathway is linked to fat synthesis by the oxidized $\rightleftharpoons$ reduced triphosphopyridine nucleotide reaction and, indeed, that oxidation of glucose by this pathway might be reduced in the absence of fat synthesis needed to provide oxidized triphosphopyridine nucleotide. In our experiments casein, lactose and fluid secretion occurred in the absence of acetate when fat secretion was much reduced.

It is possible to consider milk as a liquid resembling cell fluid (i.e. high potassium and citrate, low sodium and chloride), together with the specific milk constituents lactose, casein and fat. Histologically all the secretory cells appear to be identical, suggesting that each cell forms milk in its entirety, but it is not known to what extent the biochemical pathways controlling the formation of the various components are linked. Much recent work with isotopes shows that milk lactose, protein and fat are chiefly formed from plasma glucose, amino acids and acetate plus fat respectively. Our work with the perfused organ given variable

amounts of the three major precursors confirms this because the amount of each milk component formed is related to the amount of the precursor available. Since the formation of milk fat, lactose and protein has varied independently, this suggests that the biochemical pathways are also to a large extent independent.

Oxygen uptake and R.Q. These enabled us to compare the metabolism of the perfused goat gland with that of the sheep tissue slice reported by Folley & French (1950), who found only a small oxygen uptake and an R.Q. of less than unity with glucose only; with acetate only, both values were higher and with acetate plus glucose they were still further increased.

In our experiments the oxygen uptake and R.Q. of the perfused goat udder were low with glucose alone and markedly increased in the presence of glucose plus acetate, in agreement with the results on tissue slices. Our results with acetate alone differ, however, from the observations in tissue slices; we observed an oxygen uptake similar to that in the presence of glucose only, but an R.Q. greater than that in the presence of glucose plus acetate; in tissue slices both values with acetate alone were intermediate between those observed with glucose alone and with glucose plus acetate. These comparisons are probably valid, because Folley & French found similar values for the respiration of goat and sheep slices in the presence of acetate only.

SUMMARY

1. The influence of acetate and glucose on the respiration and milk production and composition has been studied in isolated goat udders perfused with a simplified medium and receiving adequate calories, amino acids and minerals.

2. In glands receiving glucose plus acetate the oxygen uptake and respiratory quotients were similar to those of udders of conscious goats in declining lactation, and milk, of reasonably normal composition, was secreted for 12 hr. at about 50 % of the level before the experiment.

3. Glands given glucose only had a lower oxygen uptake, and R.Q. < 1, and the milk yield was slightly reduced to about 40 % of the previous level. Milk fat concentration fell to < 20 % of the level observed in milk secreted before the experiment.

4. With acetate only, the oxygen uptake was equal to that with glucose but the respiratory quotients was higher than that observed with glucose plus acetate. However, milk secretion fell to very low levels and was restored by adding glucose.

5. Milk secretion was not stimulated in glands receiving mainly acetate by adding small quantities of glucose or fumarate.

6. Our results confirm that acetate and glucose are used to form milk fat and lactose respectively, but show in addition that glucose is essential for the secretion of fluid. It is suggested that the secretions of fluid, lactose, protein and fat are to some extent independent processes. The relationship of these findings to the pathways of glucose metabolism is discussed.

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