The use of isotopes in the study of milk secretion

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Tracers have been extensively applied in lactating animals, for reasons that are not hard to determine; it is easy to obtain samples of milk and, in dairy animals, easy to measure the yield. Thus, not only those interested primarily in mammary function, but also those interested in the passage of substances from mother to infant and in the excretion of substances from the body in milk, have resorted to the extremely simple experiment of giving a tracer amount (generally an isotope) of a substance to a lactating animal and seeing if it appears in the milk. Unfortunately it is not so easy to extract the maximum amount of information from such a convenient experiment (often, however, employing a very expensive tracer) and those who seek to do so should be reminded that there are traps for the unwary.

I cannot mention all the papers (probably hundreds) where isotopes have been used in mammary studies, but I will attempt to refer to work where isotopes have been particularly useful, or have given wrong information through misinterpretation of the data. This should be useful to nutritionists interested in using tracers in their studies. Any organ, including mammary glands, may be regarded as an organism needing to be fed. Thus one may regard what the mammary glands extract from the blood passing through them as their food, which they use partly to nourish themselves and partly to produce milk, and tracers have played an important role in identifying these compounds.

The first use of a tracer in lactation was made thirty-five years ago by the pioneer in the use of isotopes in biology, George Hevesy, (Aten & Hevesy, 1938) and, not surprisingly, the paper is a classic. They gave heavy water (D_2O) and inorganic ^{32}P to lactating goats and correctly deduced that: (1) mammary tissue is more permeable to water than to inorganic phosphate; (2) the source of milk inorganic P, casein P and ester P is the inorganic P of plasma and not plasma phosphatide P, since the specific activities (SA) of milk inorganic P and casein P were similar to the SA of plasma inorganic P and higher than that of plasma phosphatide P. They also buried the old idea that plasma phospholipids are taken up by the udder and hydrolysed, and that the lipid so released is an important precursor of milk fat, because in their experiments tissue phosphatides had a higher SA than plasma phospholipids and therefore must have been formed in the udder and not taken from plasma. Furthermore they made a useful estimate of the rate of milk secretion in terms of the time taken for milk SA to reach the initial plasma SA, which was 3–4 h.

Following this excellent start, papers began to appear in which the information obtained from the use of valuable isotope was meagre. In the instance of ^{89}Sr (Erf &

Pecher, 1940), ^{59}Fe (Erf, 1941) and ^{32}P in cows (Kleiber, Smith & Ralston, 1948) and ^{22}Na in man (Pommerenke & Hahn, 1943) the passage of these ions into milk was demonstrated, but what was reported was merely the proportion of the administered dose that was excreted in the milk; true milk SA were not reported (merely the proportion of the dose per l of milk at maximum) and blood SA were not given. In the early 1950's, when ^{14}C-labelled compounds started to become available, the use of isotopes increased greatly and by the end of the decade, when liquid scintillation counting was perfected, the only limitations to the use of isotopes were the availability of the labelled compound and the cost.

Uses of tracers

One may distinguish between the use of labelled ions (e.g. $H^{32}PO_4{}^{2-}$, $^{24}Na^+$) and labelled molecules (e.g. [^{14}C]glucose) because their uses will generally differ.

Labelled ions. All elements of milk must come from the blood, so there seems little point in documenting this unless it is important to show what proportion of an ingested dose of radioactivity is excreted in milk (e.g. ^{89}Sr from fall-out), or at what stage some ions are bound to (e.g. Ca^{2+}) or are incorporated into (e.g. P in casein) milk compounds. In the latter instance, as was done by Hevesy, one can use isotopes to determine whether incorporation occurs in mammary tissue or elsewhere, by comparing SA of product and possible precursors (see also section on labelled molecules).

Another use for labelled ions is in the study of mammary permeability to the components of the aqueous phase of milk, but again misleading results can be obtained if SA are not determined.

For example, contrary to the conclusions of my own laboratory, some workers have reported that mammary ducts actively resorb and thus alter the composition of the primary secretion to that of milk as we know it. Part of the evidence for this is that of Azimov, Orlov & Belugina (1961), who injected $H^{32}PO_4{}^{2-}$, $^{45}Ca^{2+}$ and ^{35}S into the teats of lactating goats. Following this they detected the presence of radioactivity in the blood and concluded that this demonstrated active reabsorption of phosphate, etc. However they did not measure blood or milk SA. If they had done, they would have found that during the period when radioactivity was passing into the blood most quickly, the SA of ^{32}P in milk was higher than that in blood, so that the ^{32}P atoms were merely randomly distributing themselves amongst the ^{31}P atoms. Once equilibrium is reached, the SA will be equal in blood and milk and then of course, ^{32}P will be moved at the same rate as ^{31}P and counts per ml of milk will be higher than in blood, because milk phosphate levels are higher than plasma levels. Thus isotopes cannot be used to demonstrate active reabsorption in this way.

In our experiments we inflated a pneumatic cuff around the base of a goat's teat and trapped some milk in it. Using $^{24}Na^+$, $^{42}K^+$ and $^{36}Cl^-$, we found that there was no passage of radioactivity at all from milk to blood or vice versa; a clear result. However, to show that ducts in the udder proper were also impermeable to these ions a much more complicated approach was necessary, because these ions get into milk through the secretory cells. We therefore infused into the mammary artery

for 20 min a mixture containing two of the labelled ions and, for reference, urea and ^3H_2O, and then rapidly removed all the milk in the udder in fractions and compared the SA of the ions and water in each fraction with those in the venous plasma.

The amount of ^3H_2O in each fraction of milk was proportional to its volume expressed as a percentage of the total milk in the udder (transfer quotients; Linzell & Peaker, 1971), suggesting that the permeabilities of ducts and alveoli to water were similar. However, the ions were very unevenly distributed, the transfer quotients being much higher for the alveolar milk than for the bulk milk that had been lying in the large- and medium-sized ducts. In other words, the early fractions of milk had negligible quantities of $^{24}Na^+$, $^{42}K^+$ and $^{36}Cl^-$, and the last fractions much more. Thus we concluded that at least the large ducts are impermeable to these ions from blood to milk, as the main teat duct lining is.

Labelled molecules. The main use of tracer molecules in which one or more of the constituent atoms is labelled is in the study of the metabolic pathways occurring in the tissue and in the synthesis of milk constituents and identifying milk precursors. Properly used, isotopes alone can be employed to measure quantitatively the rates of movement of chemical from one compartment of the body to another (not necessarily conforming to an anatomical compartment) and the pool size (see Zilversmit, Entenman & Fishler, 1943; Zilversmit, 1960; Atkins, 1969). Nevertheless it is advisable to combine their use with other techniques if possible.

In the measurement of the utilization of the main substrates by the body by isotope dilution (turnover or entry rates) there has been controversy as to the virtues of single shot, continuous infusion or primed continuous infusion for the most accurate results. Properly used, there is little to choose between them for the measurement of entry rates in sheep (White, Steel, Leng & Luick, 1969), although ideally a computer is needed to resolve the single shot curve into its exponential components. With continuous infusions it is not so easy to measure pool size and the extent of recycling of the infused labelled substrate. However, this can be easily done as for single shot by following the decline in SA after the infusion is stopped (e.g. see Paterson, 1964).

The impressive use of [^{14}C]acetate to demonstrate unequivocally that this is an important precursor of milk fatty acids, particularly in ruminants, is probably well known (see Folley, 1956). Success was due to the use of isotopes in vitro and in vivo, the occasional use of two isotopes simultaneously (^{13}C and ^{14}C) and the availability of quantitative non-isotopic data indicating that acetate is indeed utilized by mammary tissue in significant quantities.

Other early effective uses of isotopes in basic mammary physiology were made by Barry, who injected single doses of $H^{32}PO_4^{2-}$ and certain essential ^{14}C-labelled amino acids (Barry, 1952) and [^{14}C]glucose (Reiss & Barry, 1953) into lactating goats and followed the time course of appearance and decay of radioactivity in the plasma precursors and in milk protein and lactose respectively. His results clearly showed that these plasma substrates were the main precursors of the P and amino acids in milk protein and of both the glucose and galactose moieties of lactose, because

the SA curves of milk decayed in parallel with the plasma but 1·5–2 h later, and after 10–15 h the plasma and milk SA were equal. One can get even more informamation from such experiments. If the early part of the plasma SA curves can be delineated accurately, it is possible to deduce what proportion of the product was derived from the precursor by comparing the mean SA of precursor and product (from the integrated curves) (the 'transfer quotient'; see Kleiber, 1954).

Another excellent piece of quantitative work was carried out by Kleiber, Black, Brown, Baxter, Luick & Stadtman (1955) using two dairy cows given [^{14}C]glucose. They showed that about 20% of ^{14}C appeared in expired air in 3 h, but that half the injected dose appeared in milk and 80% of that was in the lactose. Furthermore, from the transfer quotient it was shown that 80% of lactose is derived from plasma glucose.

The value of combining tracer work with other techniques is exemplified by the simultaneous measuring of mammary uptake of milk precursors (arteriovenous differences × udder blood flow) during the measurement of entry rates by isotope dilution. This was first done by Annison & Linzell (1964) in goats and later in a pig (Linzell, Mepham, Annison & West, 1969) and cows (Bickerstaffe, Annison & Linzell, 1974). This approach at once showed that the mammary tissue itself is having a profound effect upon the lactating animal from the fact that it is removing from the circulation a large proportion of the total glucose, fatty acids and amino acids available to the whole animal. In the instance of glucose this is at least 50% and in full lactation can approach 90%. This finding led to a more widespread investigation of the effects of lactation and it was found that it produces not only growth of the liver and gastrointestinal tract but an increase in blood flow through these organs and a substantial increase in cardiac output (Chatwin, Linzell & Setchell, 1969; Hanwell & Linzell, 1973), determined by the technique of Sapirstein (1958) using ^{86}RbCl.

Another good example of the value of isotopes in elucidating a mechanism is my own work with E. F. Annison and his colleagues at Unilever, on the precursors of milk triglyceride (TG). J. M. Barry first interested me in the notion, already proposed in the 1930's, that plasma lipids (then called neutral lipids) were used by the mammary glands to synthesize milk fat. Indeed we were able to show that the goat's udder extracts large proportions of the chylomicrons and low-density lipoproteins from the plasma flowing through it, but no free long-chain fatty acids (FFA) (Barry, Bartley, Linzell & Robinson, 1963). This was confirmed by Lascelles, Hardwick, Linzell & Mepham (1964) who prepared, in other goats, chylomicrons collected from intestinal lymph, labelled with [^{3}H]stearic acid introduced into the duodenum, and infused these 'hot' chylomicrons into lactating goats and into the blood perfusing isolated lactating goats' udders. In both instances the radioactivity passed into milk fat in large quantities.

We then wondered how the mammary glands could remove so effectively such large particles (up to 1 μm), to which their capillaries would not be expected to be permeable.

The answer appeared unexpectedly from other collaborative work with E. F. Annison and his colleagues. Having measured the entry rates and mammary uptakes

of glucose and acetate in lactating goats (Annison & Linzell, 1964), we turned our attention to other substrates and used ^{14}C-labelled free palmitate, stearate and oleate. In fed animals, metabolism of these acids was very low and there were no significant net arteriovenous differences across the udder. Nevertheless there was a large transfer of radioactivity into milk fat and a large fall in SA across the udder, i.e. the FFA were being diluted with unlabelled molecules released from the udder (Annison, Linzell, Fazakerley & Nichols, 1967).

The story was completed by the infusion into the mammary artery of chylomicrons labelled differentially with ^{3}H and ^{14}C in the glycerol and fatty acid parts of the triglyceride molecules, and collecting milk, mammary venous blood and lymph during the infusion. Glycerol and fatty acids were both transferred to milk fat (although in a different ratio to that in the infused chylomicrons) and were also detected in venous blood and lymph but only in the free form (West, Bickerstaffe, Annison & Linzell, 1972). Clearly the lipids had been hydrolysed on or in the capillary wall, so that these large molecules do not pass through the capillaries as such but as the smaller, more diffusible glycerol and fatty acids. In fact mammary capillaries release a lipoprotein lipase into the venous blood (Barry *et al.* 1963) and in mice, ultrastructural studies have provided morphological evidence that chylomicrons are engulfed and hydrolysed by the endothelial cells (Schoefl & French, 1968).

Limitations in the use of labelled molecules

Barry (1952) in his early work also appreciated that when a milk component is extensively labelled from an injected isotopic precursor, this does not prove that the conversion took place in the mammary glands; it could have occurred elsewhere (e.g. in the liver or adipose tissue) with secondary uptake of the product by the mammary glands. There are 3 ways of deciding.

(a) *Searching diligently in the blood for possible precursors and determining the SA time course of both precursor and product.* Zilversmit *et al.* (1943) pointed out that, following the injection of a tracer as a single shot into the circulation, the graph of the maximum SA of a product crosses the descending curve of the immediate precursor. In the instance of a continuous infusion the SA of product will eventually equal that of the precursor, but when infusion is prolonged there is an increased chance of recycling of isotope. For example, in most isotopic studies with [^{14}C]-acetate in cows and goats the SA of all milk fatty acids up to C_{14} reach the SA of blood acetate, but C_{16} reaches only about half. However, Gerson, Sherland, Wilson & Reid (1968) showed that, after a 20 h infusion, C_{16} also reached the same SA, and concluded that this implied that all milk palmitate is synthesized in the udder, but some by a slower pathway. It may be so, but they did not check the SA of plasma free palmitate, which might have been synthesized from acetate in adipose tissue. In goats (Annison *et al.* 1967) and cows (Bickerstaffe *et al.* 1974), labelled plasma free palmitate is transferred to milk fat. Furthermore, independent data in cows shows that there is an uptake of palmitate from the plasma (arteriovenous difference × flow) which is 37% of that being simultaneously put out in the milk, implying that not more than 63% is synthesized de novo in the udder (Bickerstaffe *et al.* 1974).

Glascock (1958) has given a lucid account of the complications that can occur, which could lead the unwary to make a wrong interpretation. He gave free [³H]-stearic acid and its glyceride to cows and detected high activity in the milk fat, but could not conclude that plasma triglycerides are the precursors (which in fact they partly are) because their SA was lower than that of milk TG and much lower than that of milk FFA. Plasma phospholipids and FFA were also of low SA, so they had to search for a plasma lipid component with a SA equal to or greater than that of milk TG. They found one small fraction of the phospholipids with this property, but later it was identified by different chemical techniques as a β-lipoprotein (Glascock, Welch, Bishop, Davies, Wright & Noble, 1966), a result in agreement with our own work on goats.

This work illustrates a further point, that if an isotopically labelled compound is not administered into the compartment or body pool under study (i.e. by the intravenous or, better, the intra-arterial route in mammary studies) careful steps must be taken to identify the immediate precursor and it is always wise to remember that the compound given may be rapidly converted into the true precursor.

(b) *Use of isolated tissue (slices, homogenates, perfused glands, etc.).* It also should be remembered that blood itself is capable of carrying out some conversions. For example, white cells, and in some species (e.g. dog, man) the erythrocytes, readily oxidize glucose, so that the production of $^{14}CO_2$ from [^{14}C]glucose in a perfused dog organ would not necessarily prove that it oxidized glucose. Even milk has some cellular fragments that can convert labelled FFA into milk triglyceride (McCarthy & Patton, 1964).

(c) *Injection of the labelled compound into one gland only.* If synthesis occurs in mammary tissue the SA of a product will be higher in the injected gland, whereas if it occurs elsewhere in the body it will be equal in injected and in uninjected glands. Kleiber (1954) used this approach but injected the isotope into the teat, which is very simple. Wood, Siu & Schambye (1957) injected into the artery of one half of a cow's udder. This is the more physiological route and the SA of the injected compound is known, whereas it is uncertain after intra-mammary injection. However, surgery is required to locate the artery and this lowers the rate of milk secretion, which of course is undesirable. A way of avoiding this is to permanently exteriorize a mammary artery as a skin-covered loop to facilitate catheterization without surgery, and this has been the approach in my laboratory (Linzell, 1963, 1971).

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