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Incorporation of [*Carboxy*-^{14}C]Acetate into Lactose and Glycerol by the Lactating Goat Udder

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The ruminant udder is very active in the secretion of glycerides, and, since recent work has demonstrated that a large proportion of the milk fatty acids are synthesized from small molecules in the udder (Popják, French & Folley, 1951), the origin of the glyceride glycerol is of some interest. Experiments with slices of mammary glands have also shown that glycerol can be a limiting factor in fatty acid synthesis in mammary tissue (Balmain & Folley, 1951; Balmain, Folley & Glascock, 1952).

French & Popják (1951) obtained evidence of the conversion of glucose to glycerol in the mammary gland of the lactating rabbit. The specific activity of the glycerol isolated from the milk glycerides after administration of [^{14}C]glucose was very nearly the same as that of the lactose, and on the assumption that the latter value is a measure of the specific activity of the hexose available as substrate in the gland, it was calculated that within 6 hr. 50–100% of the glycerol was newly formed in the mamma.

Isolation of radioactive lactose from the milk secreted by the isolated bovine udder perfused with blood, containing [*carboxy*-^{14}C]acetate, has shown that the udder can incorporate acetate-carbon into lactose (Cowie *et al.* 1951). The glycerol of milk glycerides has subsequently been isolated from the experiment of Cowie *et al.* (1951), and its specific activity was found to be 10% of that of lactose, from which it follows that the udder can use acetate-carbon for glycerol formation via hexose, if not by a more direct route.

An experiment carried out by Popják *et al.* (1951) primarily with the object of studying the mechanism of the synthesis of the milk fatty acids, and in which [*carboxy*-^{14}C]acetate was injected intravenously into a lactating goat, provided an opportunity of obtaining further information about the origin of the glycerol of the milk glycerides. Lactose and glycerol have now been isolated from milk samples taken from this goat at frequent intervals after the injection, and their specific activities have been determined.

METHODS AND EXPERIMENTAL

The experimental procedure used has already been described (Popják *et al.* 1951). The amount of ^{14}C injected, as $CH_3{}^{14}COONa$, was 5 mc.

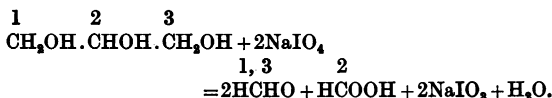
Isolation of lactose. The milk samples, from which nearly all the fat had been removed by centrifugation, were dried from the frozen state; they were stored in the cold-room for more than 2 years. Lactose was extracted from the milk samples with 70% (v/v) aqueous ethanol in a Soxhlet apparatus and was purified by recrystallization.

Isolation of glycerol. The glyceride fraction of the milk fat was saponified and, after removal of the fatty acids from the acidified hydrolysate by steam-distillation and by extraction with light petroleum (b.p. 40–60°), the aqueous phase

(also stored for more than 2 years) was neutralized with KOH and evaporated to dryness. The glycerol was extracted from the salts with boiling ethanol, the extract filtered and the ethanol removed by distillation under reduced pressure. Glycerol tribenzoate was prepared by benzoylation in water in an alkaline medium. After the benzoylated product had solidified (usually overnight at 4°), the glycerol tribenzoate was filtered off, washed with water, dried on a porous plate and crystallized twice from methanol.

Assay of ^{14}*C*. The radioactivity of glycerol tribenzoate was measured in 'infinite thickness' samples with an end-window Geiger-Müller counter (cf. Popják, 1950). Lactose was combusted in a stream of O_2 and the CO_2 collected, as described by Glascock (1951), and counted in an internal gas counter by the method of Brown & Miller (1947). In order to make the counts obtained by the two techniques comparable, a sample of the glycerol tribenzoate was also combusted and counted in the gas counter. A factor was thus obtained with which all the radioactive counts of the solid samples of glycerol tribenzoate were converted to the values of assaying the gas. Fortuitously, the radioactive counts obtained by assaying the solids had to be multiplied by a factor of only 0·99 to give the counts/mg. C as found by the gas-counting technique. The standard error of the radioactive counts of the samples up to the 12th hr. of the experiment was not more than 1%; the standard error of the counts beyond that time was 2–4%. The specific activities of both lactose and glycerol are expressed as counts/min./mg. C.

Degradation of glycerol. The periodate oxidation of glycerol was adapted for this purpose, the reaction being



$$\overset{1}{CH_2OH}.\overset{2}{CHOH}.\overset{3}{CH_2OH} + 2NaIO_4 = \overset{1,3}{2HCHO} + \overset{2}{HCOOH} + 2NaIO_3 + H_2O.$$

The formic acid, derived from C atom 2 was oxidized with HgO and the CO_2 trapped in saturated $Ba(OH)_2$ (Topper & Hastings, 1949). The formaldehyde, derived from C atoms 1 and 3, was isolated, after the oxidation of the formic acid, as the Dimedone (5:5-dimethyl*cyclo*hexane-1:3-dione) derivative (Reeves, 1941). The degradation can be carried out conveniently on 50–100 mg. of glycerol. Glycerol labelled with known amounts of ^{14}C in either C atoms 1, 3, or in C atom 2, was not available to check the specificity of the method of degradation. It has been stated, however, by Topper & Hastings (1949) that, under the conditions described by them, formaldehyde is not oxidized by HgO.

RESULTS

The specific activity/time curves for both substances are shown in Fig. 1. It will be seen that they behave exactly as predicted on theoretical grounds by Zilversmit, Entenman & Fishler (1943) for the relationship between the specific activity/time curves of a labelled precursor (in this case lactose), and its immediate reaction product (in this case glycerol). The glycerol curve is always below the lactose curve until the former reaches its maximum, when it crosses the lactose curve and thereafter declines. The specific activities of both substances decline approximately exponentially from their maxima, the half-life for lactose being estimated graphically as 2·4 hr. and for glycerol as 3·0 hr. From these values the following 'turnover' times can be calculated: lactose, 3·5 hr. and glycerol, 4·3 hr. These values imply fairly rapid rates of synthesis of both lactose and glycerol.

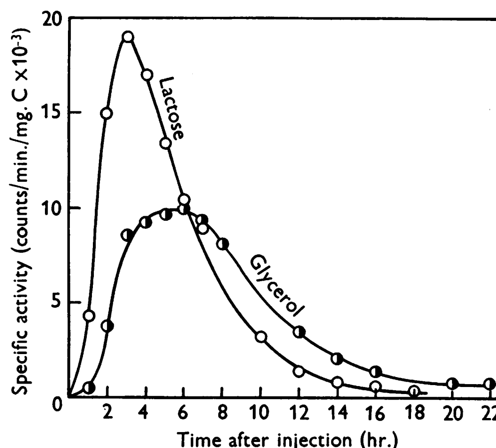


Fig. 1. Specific activities of lactose and glycerol isolated from the milk of a lactating goat which received an intravenous injection of 5 mc. of [*carboxy*-^{14}C]acetate.

A sample of the pooled glycerol was degraded to ascertain the position of the ^{14}C in the molecule. Table 1 shows that about 95% of the radioactivity of glycerol was accounted for by the isotope contents of carbon atoms 1 and 3, whereas carbon atom 2 contained only about 5% of the total ^{14}C content of the molecule.

Table 1. *Distribution of* ^{14}*C in the carbon atoms of glycerol*

(For experimental details see text.)

Material examined	^{14}C content (counts/min./mg. carbon)
Carbon atoms 1 and 3	5750
Carbon atom 2	552
All carbon atoms as determined	4050
All carbon atoms as calculated from degradation	4117

DISCUSSION

Though the results are consistent with the theory that lactose is the immediate precursor of glycerol in the udder, we do not suggest that this is actually the case. It seems more likely that glucose is the immediate carbohydrate precursor of glycerol and that lactose is in very rapid equilibrium with glucose, and, moreover, is secreted very rapidly so that its specific activity at any moment is not measurably different from that of its precursor, glucose. This also follows from the observation of

French, Popják & Malpress (1952) that the specific activities of the glucose and galactose units in lactose are identical after the administration of [^{14}C]glucose or [^{14}C]starch to lactating rabbits.

The finding of radioactive lactose in the milk after the injection of $CH_3{}^{14}COONa$ into a lactating animal must be the result of either (or of both) of two processes: (*a*) incorporation of the isotope into blood glucose (in the liver) and transport to the udder; and (*b*) synthesis of hexose within the udder itself. Our experiment provided no information on the first of these processes. However, significant amounts of radioactivity are found in the blood glucose of lactating rabbits after the injection of either [^{14}C]acetate or of [^{14}C]pyruvate. On the other hand, the incorporation of acetate-carbon into lactose in the isolated udder has been shown to occur by a mechanism other than carbon dioxide fixation (Cowie *et al.* 1951) and, therefore, it seems probable that in our experiment on the intact animal the appearance of radioactive lactose was due, at least partly, to synthetic activities within the udder itself. The amount of incorporation of acetate-carbon into lactose and glycerol was fairly large as judged by the specific activity of both substances. The maximum radioactivities of the lactose and glycerol shown in Fig. 1 correspond to about $9{\cdot}5 \times 10^{-3}\ \mu$c. ^{14}C/mg. carbon and $5 \times 10^{-3}\ \mu$c. ^{14}C/mg. carbon respectively. It is of interest that the highest radioactivities of the long-chain saturated and unsaturated fatty acids in the milk of this animal (observed 4 hr. after the injection of acetate) were $20 \times 10^{-3}\ \mu$c./mg. carbon and $6 \times 10^{-3}\ \mu$c./mg. carbon respectively, while the maximum radioactivity of the short-chain fatty acids was $70 \times 10^{-4}\ \mu$c./mg. carbon (cf. Popják *et al.* 1951, Fig. 3).

The metabolism of acetate by the citric acid cycle, which is known to operate in the mammary gland (Terner, 1951), provides a path for the entry of acetate-carbon into pyruvate, and hence, by the reversal of the glycolytic cycle, acetate-carbon can find its way into glucose (cf. Wood, 1948; Topper & Hastings, 1949). It appears that in our experiment too, ^{14}C from $CH_3{}^{14}COONa$ was incorporated into hexose by the same process. It is known that after the administration of $CH_3{}^{14}COONa$ the glucose units of liver glycogen contain practically all the isotope in carbon atoms 3 and 4 (Wood, 1948; Topper & Hastings, 1949). This is accounted for by the fact that the metabolism of [*carboxy*-^{14}C]acetate by the citric acid cycle yields pyruvate labelled in the carboxyl carbon which will enter into positions 3 and 4 of glucose. The breakdown of [3:4-^{14}C]-glucose into glycerol should yield [1:3-^{14}C]glycerol. The results of the degradation of glycerol shown in Table 1 are in complete harmony with this conception. The finding of some radioactivity in carbon atom 2 of glycerol indicates that positions 2 and 5 of the hexose must have also contained some ^{14}C.

In our experiment it is not certain whether the liver or the udder is the most likely site for the incorporation of acetate-carbon into glucose; the data shown in Fig. 1, however, make it seem very likely that the breakdown of glucose to glycerol occurs within the udder itself. Glycerol could be formed from glucose at the triosephosphate stage of the glycolytic breakdown. Dismutation of triosephosphate would yield glycerophosphate which could be dephosphorylated by the very active phosphomonoesterase present in mammary tissue (Folley & Kay, 1935). If lactose synthesis from small molecules occurs appreciably within the udder it might be argued that glycerol should appear as the precursor of lactose, since triosephosphate (presumably in equilibrium with glycerol) is an intermediate between pyruvate and hexose. The most likely explanation of the fact that lactose appears as the precursor of glycerol, and not the other way about as this argument would require, seems to be that the reaction triosephosphate $\rightarrow$ glycerol is much slower than triosephosphate $\rightarrow$ hexose, or else that the contribution to the milk lactose of radioactive glucose formed in the liver and carried to the udder in the blood must have greatly predominated over that of radioactive glucose formed in the gland itself. Further work is in progress to elucidate in detail the mechanisms of hexose formation in the mammary gland from various substrates.

While it may have been reasonably anticipated that glycerol in mammalian tissues is derived from glucose, just as in the fermentation of glucose by yeast, we do not know of any formal proof of this reaction in animals, other than that recorded by French & Popják (1951) and in this article.

SUMMARY

1. The specific activity/time curves for lactose and glyceride glycerol in the milk of a goat injected with [*carboxy*-^{14}C]acetate exhibit the mutual relationship characteristic of the curves for a labelled precursor (lactose curve) and its immediate reaction product (glycerol curve).

2. It is concluded that glucose (from which lactose is rapidly formed) is the carbohydrate precursor of glycerol in the udder.

3. The distribution of ^{14}C in the glycerol molecule (95 % in carbon atoms (1) and (3)) is consistent with the view that the acetate-carbon entered glucose by way of the citric acid cycle followed by the glycolytic process in reverse.

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The Hydrolysis of Succinyldicholine by Cholinesterase

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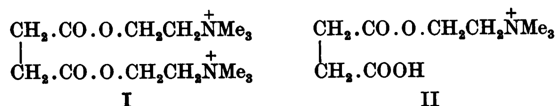
The dicholine ester of succinic acid (I) has recently found clinical application as a powerful curare-like drug with a short-lasting action and relative freedom from undesirable reactions (Bovet, Bovet-Nitti, Guarino, Longo & Marotta, 1949). Unlike natural curare, its action is potentiated, not antagonized, by eserine, and since it is also known to be hydrolysed *in vitro* by plasma cholinesterase (Glick, 1941; Bovet-Nitti, 1949), its transient action is probably due to destruction by cholinesterase *in vivo*. As the half ester (II) is without



curare-like action, inactivation could conceivably be brought about either by simultaneous hydrolysis of both links of (I), giving a mixture of choline and succinic acid, or by hydrolysis to (II) as the intermediate or final product. The mechanism of the destruction of succinyldicholine by cholinesterase is thus of interest both from the pharmacological and enzymological points of view. We have studied the problem *in vitro* by means of chromatographic separation of the constituents of the reaction mixture at different stages of the hydrolysis. In earlier work (Whittaker, 1951) the solvent system used effected the separation of the two esters of succinic acid but not that of succinylmonocholine and choline, and these could only be distinguished by means of a reagent giving a coloration with carboxylic esters. A solvent mixture has now been found which gives good separation of all four of the possible constituents of the reaction mixture, namely succinyldicholine, succinylmonocholine, choline and succinic acid, and the chromatographic studies have been supplemented by a kinetic analysis, using the Warburg technique.

METHODS

Source of enzyme. Horse serum cholinesterase was prepared by the method of Strelitz (1944) from commercial horse serum. The preparation corresponded to that obtained at stage 3 of her procedure and had initially a specific activity of 33 120 μl./ml. enzyme solution/hr., measured manometrically at pH 7·4 and 38° with acetylcholine (30 mM) as the substrate. The enzyme was stored at 0° with $CHCl_3$ as a preservative; its activity was unchanged by incubation without added substrate for several hours at 38°, but slowly declined during the course of the work to 23 600 units. Initial velocities are expressed as μmoles acid liberated/3 ml./hr. in presence of 23 600 units of enzyme activity.

Substrates. The choline esters and choline were employed as the perchlorates.

Paper chromatography. This was carried out as described in a previous paper (Whittaker & Wijesundera, 1952). Immersion in I_2 vapour (Brante, 1949; Marini-Bettolo-Marconi & Guarino, 1950) proved a useful general method, giving yellowish or brown spots with choline and its esters and succinic acid. The carboxylic ester reagent (Whittaker & Wijesundera, 1952) was used to detect the succinylcholine esters, and bromothymol blue, used as described by Brown (1950), detected succinylmonocholine and succinic acid as yellow spots. Whatman no. 4 filter paper was used throughout.

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