

Utilization of Acetate for Milk-fat Synthesis in the Lactating Goat

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(Received 1 September 1950)

The experiment to be reported is concerned mainly with two related problems of milk secretion: (a) with the origin of the glyceride fatty acids in the milk, and (b) more specifically, with the origin of the short-chain (steam-volatile) fatty acids which are found in the milk of many species, particularly of herbivorous animals, but which have not been detected in body fat.

The subject has been recently reviewed (Folley, 1949) and here only the main features of current views will be summarized. Graham, Jones & Kay (1936) concluded that the amount of glyceride fat which disappeared from the blood during passage through the udder of lactating cows was sufficient to account for the production of milk fat. Voris, Ellis & Maynard (1940), using a more specific method for the estimation of blood glycerides, confirmed the uptake of blood fat by the udder of lactating cows. On the other hand, the finding of a high respiratory quotient for the active udder *in vivo* led to the suggestion that part of the milk fat might be synthesized in the gland from carbohydrate sources (Graham, Houchin, Peterson & Turner, 1938). To explain the occurrence of the short-chain acids several speculative views have been put forward. It was suggested, for instance, by Reineke, Stonecipher & Turner (1941), that if part of the milk fat is synthesized in the gland from carbohydrate, the short-chain acids might be the part so formed. The most fully developed theory, however, that of Hilditch, assigns no synthetic role to the glandular tissue, but points to the blood glycerides, especially their oleic acid component, as the source of the volatile acids (see Hilditch, 1947, pp. 306-10).

Evidence of a more direct kind has appeared recently from studies of acetate metabolism in mammary tissue. On the basis of *in vitro* utilization of acetate by lactating mammary gland slices, with an R.Q. greater than one, Folley & French (1948, 1950) inferred that fatty acid synthesis from acetate occurred in these slices, and that the short-chain acids may be intermediate products. Popják & Beeckmans (1949, 1950) have demonstrated the synthesis of fatty acids and of cholesterol in the mammae of non-lactating pregnant rabbits with the aid of deuterium and ^{14}C -labelled acetate. A particularly high radioactivity was found in the glyceride

fatty acids of the mammae after the injection of the labelled acetate. Further work showed that this high radioactivity was due to the presence of volatile fatty acids of exceptionally high ^{14}C content (Popják, Folley & French, 1949; Popják & Beeckmans, 1950). Since the volatile acids contained seven to eighteen times more ^{14}C than the long-chain acids in either the mammary gland or the liver, it was concluded that the volatile acids are not derived from the degradation of the long-chain acids, but from the condensation of C_2 units within the gland.

In the experiments on pregnant rabbits, the fat extracted from the mammae contained volatile acids, and to this extent was comparable to milk fat, but the extent of its admixture with tissue fat could not be ascertained. In continuation of these observations it was desirable to obtain adequate quantities of true milk fat for detailed study over a period which allowed the time course of the synthetic process to be followed.

Although the investigation of acetate metabolism in the mammary gland has had the effect of linking up the problem of milk-fat formation with the wider aspects of fat metabolism, in which acetate has a central role (see Bloch, 1947, 1948), another aspect of acetate metabolism had to be kept in mind in planning this experiment. In ruminants, the importance of acetic acid, formed by fermentation of cellulose and other polysaccharides in the rumen, as one of the main forms in which carbohydrate food is assimilated, has been established by Barcroft and his collaborators (see review by Elsdon & Phillipson, 1948). Folley & French (1950) have found species differences in the metabolism of mammary gland slices, which they interpret as indicating the preferential use of acetate rather than glucose for milk-fat synthesis in ruminants. Therefore both the quantitative and qualitative aspects of acetate metabolism have special interest for ruminant physiology. The present experiment on a lactating goat, apart from giving information on milk fat formation, which may or may not be valid for other species, afforded also an opportunity to study other aspects of acetate metabolism in the ruminant, such as the rate of its oxidation.

A preliminary communication of this work has already been given (Popják, French & Folley, 1950).

MATERIAL AND METHODS

A lactating pedigree goat (117 lb., 53 kg.) was the experimental animal. First the udder was emptied as completely as possible by hand-milking after intravenous injection of oxytocin, then an isotonic solution of [carboxy- ^{14}C] acetate was injected into the jugular vein. The total ^{14}C dose was 5 mc. contained in 430 mg. of anhydrous sodium acetate. The goat was kept under cover in the field during the whole of the experiment.

Collection of respiratory CO_2 . Immediately after the injection, the head of the goat was put into a respiration chamber constructed from the 'astrodome' (a plastic half-sphere) of a bomber aircraft. The 'astrodome' was fastened airtight to a vertical perspex sheet backed by a wooden board with a hole in the centre sufficiently large to take the head of the goat. A rubber collar, fastened to the hole, fitted snugly around the shaved neck of the goat and was secured further to the neck with adhesive tape. A brisk stream of air was drawn into the chamber with the pump of a milking machine through a soda-lime tower. The respiratory CO_2 was trapped in 10N-NaOH solution placed in two trains of absorbers, each consisting of six bottles in series. The two trains were used alternately as the renewal of the absorbant required. The respiratory CO_2 was collected continuously for 6 hr. after which the goat was removed from the respiration chamber. Representative samples were taken 15, 30 min., 1, 2, 3, 4, 5 and 6 hr. after the injection and the CO_2 was precipitated as BaCO_3 for assay of ^{14}C .

The goat showed no signs of discomfort whatever, but stood placidly with its head in the respiration chamber.

Collection of milk. The animal was milked by hand hourly during the first 8 hr. of the experiment, then 2-hourly during the following 6 hr., 3-hourly during the next 12 hr., and twice more at 6 hr. intervals during the remaining 12 hr. of the experiment. The average yield of milk was 100 ml./hr. The assistant who milked the animal wore rubber gloves and a gas mask fitted with a soda-lime canister.

The experiment lasted for 48 hr. when the animal was killed by bleeding from the carotid artery under nembutal anaesthesia. Blood and viscera were taken for extraction of lipids. Blood was drawn also at 6 and 24 hr. after the injection of acetate from the jugular vein on the opposite side to the one into which the labelled acetate was injected. The blood samples were used for the extraction of plasma lipids.

Extraction of lipids and fractionation of fatty acids. The milk fat was first separated by centrifuging, after which the centrifuge tubes were iced. The fat was then lifted out of the tubes as a solid pad. The milk-fat samples were kept refrigerated until extraction, which was completed for all twenty-two samples within 48 hr. after the end of the experiment.

The viscera were pulped in a Waring blender and were dried from the frozen state *in vacuo*. The methods of extraction and fractionation of lipids, except for the separation of volatile fatty acids, were those already described (Popják & Beeckmans, 1950). The volatile fatty acids from the milk and udder glycerides were obtained after saponification as described by Hilditch (1947, p. 467). They were further divided into those soluble and insoluble in water at 0°. The acids insoluble in water were filtered off and washed on the filter paper with iced water. The acids remaining on the paper were dissolved in hot ethanol. Both the water soluble

and insoluble fractions were neutralized with NaOH and were evaporated to dryness on the water bath. The non-volatile long-chain acids were separated into solid and liquid acids by the lead salt method (Twitchell, 1921). The phospholipins of milk were not examined.

Assay of ^{14}C was carried out on the Na salts of fatty acids in infinite thickness (>25 mg./sq.cm.) samples as described previously (Popják, 1950). Because of the unexpectedly high ^{14}C content of many of the fatty acid fractions, the samples for assay were prepared on disks of 1 sq.cm. area. With the Geiger-Müller counter used and the particular geometrical conditions of assay a substance containing $1 \times 10^{-3} \mu\text{c.}$ of $^{14}\text{C}/\text{mg.}$ gave about 1000 counts/min. in an infinitely thick sample of 1 sq.cm. area. All counts were corrected for background and for counts lost due to the dead-time of the quenching circuit used.

RESULTS

Oxidation of acetate

Fig. 1, curve A, shows the specific activity:time curve of the respiratory CO_2 . The specific activities are shown as $\mu\text{c. } ^{14}\text{C}/\text{mg.}$ carbon. The maximum

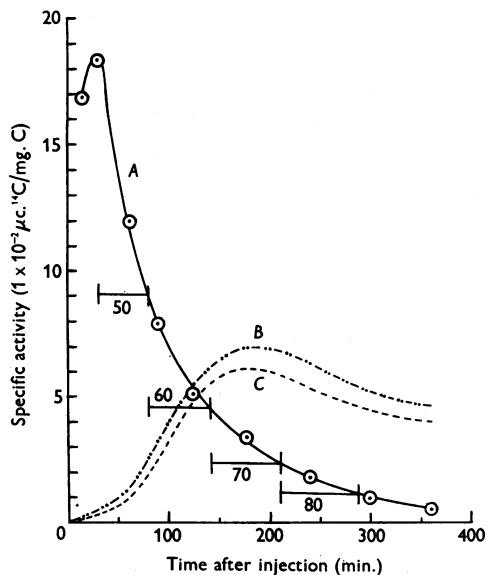


Fig. 1. Specific activity:time curves of respiratory CO_2 , and of water-soluble and water-insoluble steam-volatile fatty acids of milk after the injection of 5 mc. of ^{14}C as $\text{CH}_3^{14}\text{CO}_2\text{Na}$ into a lactating goat. A, respiratory CO_2 ; B, water-insoluble steam-volatile fatty acids; C, water-soluble steam-volatile fatty acids. The horizontal bars with the figure beneath indicate the half-life (in min.) of the specific activity:time curve of the respiratory CO_2 at different periods of the experiment.

excretion of acetate carbon as CO_2 was reached 15–30 min. after the injection and was followed by a steady fall in the specific activity of the CO_2 . Curve A in Fig. 1 is not a simple exponential curve;

this is apparent from the fact that it does not decay with a constant half-life. The half-life of this curve increased from 50 min. during the first hour of the experiment to 80 min. during the fifth and sixth hours as indicated by the horizontal bars in the figure. It seems probable that the initial high specific activity of the respiratory CO_2 was due mainly to the oxidation of acetate, but later, as the injected acetate became incorporated into other organic substances, the oxidation of the latter provided additional radioactive CO_2 . From the initial portion of this curve it is estimated that the half-life of body acetate in the goat under the conditions of the experiment is not more than 50 min., probably less.

By graphical integration of curve A, Fig. 1, it has been calculated that the average specific activity of the respiratory CO_2 was $0.67 \mu\text{c./mmol}$. The total weight of barium carbonate collected in 6 hr. from the respiratory CO_2 was 1.2 kg., which is just over 6 g.mol. Thus, assuming that the goat produced 1 g.mol. of respiratory CO_2 /hr., 80% of the injected acetate was completely oxidized in 6 hr. This is a lower value than that reported for rats (87% excretion in 4 hr.) by Gould, Sinex, Rosenberg, Solomon & Hastings (1949). Also the half-life of the respiratory $^{14}\text{CO}_2$ in rats was only 25 min. between 40 and 120 min. after the intraperitoneal injection of [*carboxy*- ^{14}C] acetate (Gould *et al.* 1949). Thus it seems that the rate of oxidation of injected acetate in the lactating goat is considerably slower than in the rat. This observation might well be related to the extremely high degree of utilization of acetate for synthetic reactions in our animal, as will appear below. Whether this phenomenon applies in general to ruminants, or only to lactating ruminants, is unknown at present.

The two interrupted curves, B and C in Fig. 1, show for the sake of comparison the specific activity-time curves of the steam-volatile fatty acids obtained from the milk; these curves are shown with the experimental points in greater detail in Fig. 3.

The sources of glyceride fatty acids and cholesterol in milk

In order to obtain evidence on the question whether or not milk fatty acids and cholesterol are derived primarily from the plasma lipids or are synthesized in significant amounts in the udder, the ^{14}C contents of these substances in the plasma were determined and compared with those in the milk. The results are shown graphically in Figs. 2 and 3. The ^{14}C content of the plasma fatty acids differed so greatly from that of the milk fatty acids, that the comparison cannot be made on one graph. It should be pointed out that the scale of the ordinate in Fig. 2 is 200 times larger than in Fig. 1 and 100 times larger than in Fig. 3.

The maximum ^{14}C content of milk cholesterol was

reached 12 hr. after the injection of acetate, and was followed by a steady decline in spite of the fact that the specific activity of plasma cholesterol continued to rise. Such a relationship can only mean that milk cholesterol is not derived from the blood, but is synthesized within the udder from available precursors. If milk cholesterol were obtained from the blood its specific activity should rise until it reaches that of plasma cholesterol and should decline only afterwards. The theoretical basis for this type of correlation between precursor and product is given by Zilversmit, Entenman & Fishler (1943).

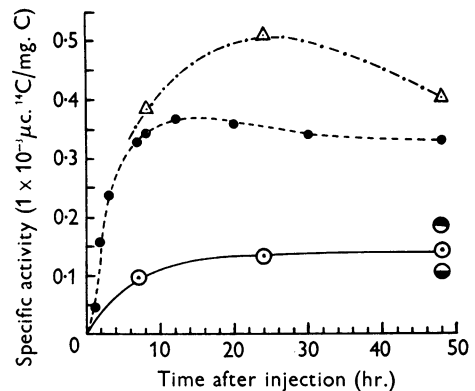


Fig. 2. Specific activity-time curves of plasma (Δ) and milk cholesterol (\bullet) and of plasma total fatty acids (\circ) after the injection of 5 mc. of ^{14}C as $\text{CH}_3^{14}\text{CO}_2\text{Na}$ into a lactating goat. \bullet , Specific activity of phospholipin; \circ , specific activity of non-phospholipin fatty acids at the end of the experiment.

The rise in ^{14}C content of plasma total fatty acids became very slow 24 hr. after the injection, but continued up to the end of the experiment (48 hr.). Since at the time of killing the animal large amounts of plasma were available, phospholipin and non-phospholipin fatty acids and total fatty acids of the plasma were assayed for their ^{14}C content. Fig. 2 shows that the phospholipin fatty acids contained more ^{14}C than the non-phospholipin fatty acids and that the specific activity of the total fatty acids was about the mean of the two. No conclusion can be drawn from the observed difference between the phospholipin and non-phospholipin fatty acids. It has been pointed out by Phil & Bloch (1950) that the non-phospholipin fatty acids in plasma contain a large proportion of fatty acids originally present in free form and also fatty acids derived from cholesteryl esters and that the latter are known to consist to a large extent of essential fatty acids. It is therefore likely, as in the experiments of Phil & Bloch (1950), that the plasma glyceride fatty acids contained more ^{14}C than the figure for the non-phospholipin fatty acids would indicate.

The milk fatty acids behaved quite differently from the plasma fatty acids (Fig. 3). The steam-volatile fatty acids reached their maximum specific activity 3 hr. and the non-volatile acids 4 hr. after the injection, when they contained several hundred times more ^{14}C than the plasma fatty acids did at any time. If the specific activity:time curve of the plasma fatty acids were superimposed on the first part of the graph shown in Fig. 3 (up to 12 hr. after the injection of the labelled acetate), this curve would not be above the level of the abscissa by more than the thickness of the line.

first 6 hr. of the experiment. It has been calculated from the graphical integration of the specific activity:time curves of the milk fatty acid fractions and from the average yield of the four crude fatty acid fractions, that during the same 6 hr. 500 μc . of the injected acetate appeared as milk fat, i.e. 10% of the total injected dose, or 50% of the amount of acetate retained in the body.

The specific activity:time curves of all the milk fatty acids decayed with a half-life of about 4 hr. throughout the experimental period. This indicates an extremely rapid rate of synthesis, in all prob-

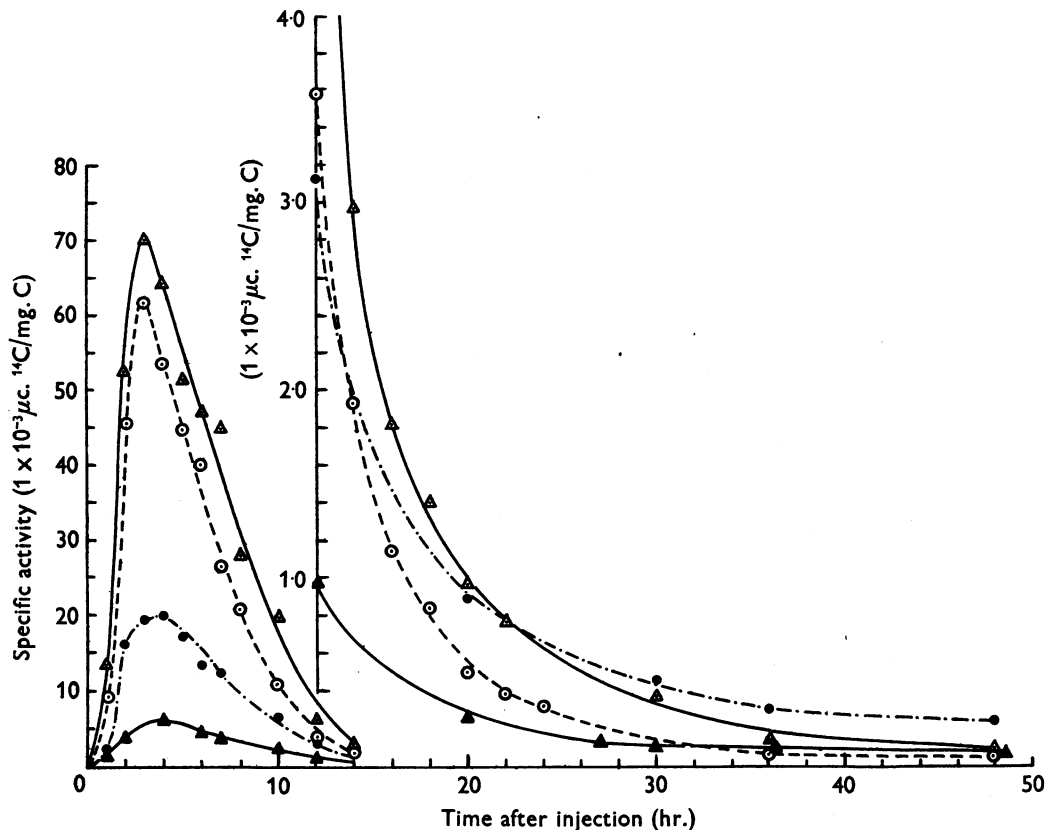


Fig. 3. Specific activity:time curves of four crude fatty acid fractions of goat milk after the injection of 5 mc. of ^{14}C as $\text{CH}_3^{14}\text{CO}_2\text{Na}$. \circ --- \circ , Water-soluble steam-volatile fatty acids; \triangle — \triangle , water-insoluble steam-volatile fatty acids; \bullet --- \bullet , solid, non-volatile, fatty acids; \blacktriangle — \blacktriangle , liquid, non-volatile, fatty acids.

This gross comparison between plasma and milk fatty acids indicates clearly that synthesis of fatty acids in the udder from small molecules (e.g. acetate or some other C_2 unit) is of outstanding importance in the origin of milk fat.

The magnitude of acetate utilization for milk-fat synthesis can be best judged from the following consideration. It has already been mentioned that of the injected 5 mc. of ^{14}C as acetate, 80%, i.e. 4 mc., appeared in the respiratory CO_2 during the

ability equal to the rate of secretion of fatty acids in the milk.

Another important point emerges from the relationship between the specific activity:time curves of the short-chain volatile acids and those of the non-volatile (solid and liquid) acids. At the time of their maxima the former contained several times more ^{14}C than the latter, and therefore it must be concluded that the short-chain acids have originated by synthesis from acetate (or an active derivative of

it) and not by degradation of oleo-glycerides, thus confirming the previously quoted results obtained on non-lactating pregnant rabbits (Popják *et al.* 1949; Popják & Beeckmans, 1950).

In Table 1 are recorded the specific activities of the fatty acids and cholesterol obtained from the liver, udder and milk of the animal at the end of the experiment. It should be made clear that such comparisons, especially between the liver and udder, may have very little significance because the composition of the fatty acids is totally different in the two organs.

Table 1. ^{14}C Content of fatty acids and cholesterol in liver, udder and milk of goat 48 hr. after the injection of 5 mc. ^{14}C as $\text{CH}_3^{14}\text{CO}_2\text{Na}$

(^{14}C content expressed as 1×10^{-4} $\mu\text{c.}/\text{mg.}$ substance.)

	Liver	Udder	Milk
Glyceride fatty acids			
Steam-volatile	—	—	—
Water-soluble	—	1.37	0.31
Water-insoluble	—	2.93	0.70
Non-volatile	—	—	—
Solid	0.36	3.77	1.94
Liquid	0.48	1.32	0.68
Phospholipin fatty acids			
Solid	2.27	2.04	—
Liquid	0.94	0.52	—
Cholesterol	3.62	2.80	2.80

Even the fact that the glyceride fatty acid fractions in the udder contained more ^{14}C than in the liver cannot be interpreted as signifying a greater utilization of acetate for fatty acid synthesis in the former than in the latter organ (although this is very probable), because these values represent only single points on a time curve. The higher specific activities of the udder fatty acids as compared with those of the milk might be attributed to a retention of fat from earlier periods of the experiment in some parts of the gland which are not completely emptied by milking.

DISCUSSION

In the interpretation of the results described it has to be borne in mind that the four fatty acid fractions were not homogeneous. The steam-volatile acids soluble in water, for example, contain acids from C_4 to C_8 and those insoluble in water from C_8 to C_{12} (and even traces of C_{14} and C_{16}) chain length. Particular caution must be exercised in the case of the non-volatile saturated and unsaturated acids as separated by the lead-salt method. This technique is fairly efficient when the separation of saturated and unsaturated acids of C_{16} or longer chain-length is desired. In the fat of goat milk, however, appreciable amounts of dodecanoic (lauric) and tetradecanoic (myristic) acids are present, the lead salts of which are fairly soluble, and therefore a significant

portion of these acids appears in the unsaturated fraction (cf. Hilditch, 1947, p. 488). It is therefore quite possible that the crude fatty acid fractions contain individual acids perhaps of widely different isotope content, the knowledge of which is essential for the complete interpretation of our findings.

In particular, the fact that the non-volatile acids are synthesized to a considerable extent in the mammary gland, as shown by their ^{14}C content, raises the question whether the shorter-chain acids are intermediate products of the condensation of C_2 units, which, by the process of rapid secretion into the milk, have escaped further elongation. In this connexion it may be noted that the differences in height of the specific activity: time curves of the four fatty acid fractions do not bear a simple relationship to the average chain-length of their components. These curves all decay with the same half-life (about 4 hr.) in spite of the inhomogeneity of the fractions and their different specific activities. It seems probable that these relationships reflect the biochemical mechanisms of synthesis and it is hoped that the isotope content of the pure acids, which will be published in a separate paper, will throw light on this question. It can be stated already, however, that pure oleic acid as obtained from the milk had a relatively low isotope content, so that it could not have been the main source of the shorter-chain acids.

The uptake of blood fat by the udder as shown by the measurement of arterio-venous differences (see Folley, 1949) may be related in two ways to the synthetic process demonstrated in this experiment. In the first place the transfer of pre-formed fatty acids of low isotope content from blood to milk may account, at least partly, for the different specific activities of the milk-fat fractions. Secondly, it must be emphasized that the results do not warrant the assumption that all the acetate used for milk-fat synthesis is derived as such from the blood. The acetate from which the udder synthesizes fatty acids may have a dual origin: (a) exogenous, obtained from the rumen via the blood (McClymont, 1949) and (b) endogenous, from the breakdown of higher fatty acids absorbed from the blood. The acetate from these two sources could not be distinguished in our experiment.

The dynamic equilibrium of cell constituents, maintained by the balance between synthesis and breakdown, is now well established for internal organs on the basis of isotope experiments. This type of endogenous metabolism probably applies to the mammary gland also, but in a form modified to allow for the rapid secretion of the synthetic products. The extent to which breakdown occurs in the udder is at present quite uncertain; but as far as the formation of milk fat is concerned it seems clear that it results from the endogenous metabolism of the mammary cells to a greater extent than was formerly supposed.

SUMMARY

1. The oxidation of acetate and its utilization for the synthesis of milk fatty acids in a lactating goat has been investigated with the aid of [carboxy-¹⁴C] acetate.

2. The half-life of body acetate in the lactating goat is not more than 50 min.

3. Acetate is rapidly utilized for the synthesis of milk fatty acids; the specific activity: time curves of these, after the maxima had been attained 3–4 hr. after the injection, decayed with a half-life of about 4 hr.

4. The plasma fatty acids contained much less ¹⁴C than the milk fatty acids and could not have been directly an important source of the latter.

5. The steam-volatile fatty acids had a higher specific activity than the long-chain non-volatile acids indicating their independent synthesis. The hypothesis that the volatile fatty acids of milk originate from the degradation of long-chain acids (particularly of oleic) could not be confirmed. They may represent intermediates in the formation of long-chain acids.

6. Milk cholesterol is synthesized in the udder and not derived from blood cholesterol.

Our thanks are due to Mr S. C. Watson for the design and construction of the respiration chamber and for his generous assistance during the experiment even at night time, to Dr A. T. Cowie for taking the blood samples and dissecting the animal and to Misses M. Beard and P. Taylor for milking and taking care of the goat.

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The Amylase of *Clostridium butyricum*

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(Received 22 June 1950)

Baker & Nasr (1947) demonstrated by microscopical observation that the breakdown of raw potato starch in the caecum of the pig is caused by large iodophile sporing rods exercising their action *in situ* upon the surface of the starch granules. The micro-organism was subsequently isolated in pure culture and identified as a strain of *Clostridium butyricum* (Baker, Nasr & Morrice, 1948). The pure culture was able to break down raw potato starch granules *in vitro*, the morphological and histochemical features of the process being similar to those observed in

granules present in the caecal contents of pigs fed on potato starch diets. Evidence was presented (Nasr & Baker, 1949) which indicated that the extracellular decomposition of starch and other carbohydrates was accompanied by an intracellular synthesis of iodine-staining polysaccharide operated by a phosphorylating mechanism. The presence of amylase was demonstrated in cell-free culture filtrates when the organism was grown on starch. The isolation of this amylase in a stable solid state and an examination of its properties form the subject