Palade, G. E. (1956). *J. biophys. biochem. Cytol.* **2**, 85.
Roth, J. S. (1954). *Nature, Lond.*, **174**, 129.
Russell, J. A. (1951). *Endocrinology*, **49**, 99.
Russell, J. A. (1955). In *Hypophyseal Growth Hormone, Nature and Actions*, p. 213. Ed. by Smith, R. W., jun., Gaebear, O. H. & Long, C. N. H. New York: McGraw-Hill.
Rutman, J., Rutman, R. J. & Tarver, H. (1955). *J. biol. Chem.* **212**, 95.
Sachs, H. (1957). *J. biol. Chem.* **228**, 23.
Scott, J. F., Fraccastoro, A. P. & Taft, E. B. (1956). *J. Histochem. Cytochem.* **4**, 1.
Siekevitz, P. (1952). *J. biol. Chem.* **195**, 549.
Simpson, M. E., Evans, H. M. & Li, C. H. (1949). *Growth*, **13**, 151.
Ulrich, F., Tarver, H. & Li, C. H. (1954). *J. biol. Chem.* **209**, 117.
Weiss, P. (1955). In *Hypophyseal Growth Hormone, Nature and Actions*, p. 3. Ed by Smith, R. W., jun., Gaebler, O. H. & Long, C. N. H. New York: McGraw-Hill.
Wilhelmi, A. E., Fishman, J. B. & Russell, J. A. (1948). *J. biol. Chem.* **176**, 735.
Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.

Incorporation of Sodium [1-^{14}C]Hexanoate and Sodium Hydrogen [^{14}C]Carbonate into Milk Constituents by the Perfused Cow's Udder

By MONIQUE LAURYSSENS, R. VERBEKE, G. PEETERS and AGNES DONCK
Physiological Department of the Veterinary College, University of Ghent, Belgium

(*Received* 7 *January* 1959)

The occurrence of a C_6 volatile acid was observed in the ox rumen by McClymont (1951) and in the sheep rumen by Gray, Pilgrim, Rodda & Weller (1952). The metabolism of [1-^{14}C]hexanoate and [2-^{14}C]hexanoate in the intact dairy cow was studied by Kleiber and his collaborators. Most of the ^{14}C activity, recovered in milk constituents, was present in the lactose, smaller quantities of ^{14}C being found in the fat and casein. The specific activity of plasma glucose, after injection of [1-^{14}C]hexanoate, was much higher than that of acetate. It was concluded from this observation that the transfer of carbon derived from hexanoate to glucose mostly bypasses the plasma acetate pool, even though acetate can contribute carbon to plasma glucose. A similar conclusion was reached from the labelling of plasma acetate and glucose after injection of ^{14}C-labelled butyrate (Kleiber, 1958).

In the experiments described here we have studied the incorporation of sodium [1-^{14}C]hexanoate into different constituents of milk and tissue by the perfused cow's udder. The results indicate that the udder has the capacity to degrade hexanoate to acetyl-coenzyme A. This hexanoate does not behave in a glycogenic manner.

In a previous experiment it was shown that $^{14}CO_2$ was incorporated only to a negligible extent into fatty acids and lactose by the perfused-udder preparation (Cowie *et al.* 1951). No information is available about the incorporation of carbon dioxide into the amino acids of casein by the same preparation. For these reasons a perfusion experiment was performed in the presence of sodium hydrogen [^{14}C]carbonate and the incorporation into amino acids was studied.

EXPERIMENTAL

Perfusion of half-udders. Each experiment was carried out on one lactating half-udder perfused during 150 min. by the method of Peeters & Massart (1952). Half-udders from different cows were utilized. Experimental details of the perfusion technique and of $^{14}CO_2$ collection were as described by Verbeke, Lauryssens, Peeters & James (1959). Immediately before slaughter the cows were milked out as completely as possible with the aid of intravenously injected oxytocin. The udders were removed as soon as the cows were shot. The glands were completely bisected along the median septum and one half was connected to the perfusion apparatus. Heparinized, fresh cow's blood (9 l.) was used for the perfusion of one half.

Sodium [1-^{14}C]hexanoate (0·2 m-mole = 1 mc) was dissolved in 50 ml. of water and added to the blood 30 min. after the beginning of perfusion. The experimental animal had yielded approx. 18 l. of milk daily. After the administration of labelled substrate, inactive acetate (sodium acetate trihydrate, 5·25 g. in 250 ml. of water) was introduced regularly into the blood reservoir by means of a constant-drip device. At the end of the experiment oxytocin (10 i.u.) was injected into the udder artery to cause ejection of milk: 290 ml. of milk was collected. Skin, teats and adipose tissue were removed carefully by dissection. The udder tissue was weighed (wt. 3·88 kg.), cut into pieces and cooled at $-15°$ for 1 hr.

Sodium hydrogen [^{14}C]carbonate (5·9 mg. = 1 mc) was added to a solution of inactive sodium acetate trihydrate (10·5 g. in 750 ml. of water). This solution was added to the blood reservoir by means of a constant-drip device started 30 min. after the commencement of perfusion. The experimental animal had previously yielded approx. 12 l. of milk daily. After perfusion 150 ml. of milk was collected.

Isolation of udder tissue and milk-fat fractions. Udder tissue and milk-fat fractions were isolated after perfusion with [1-^{14}C]hexanoate as described previously (Verbeke *et al.* 1959).

Table 1. *Specific activities of constituents of udder tissue and milk after perfusion with* $NaH^{14}CO_3$ *or* [1-^{14}C]*hexanoate*

Specific activities are given as mμc/mg. of C (±S.D.).

Substance	Udder tissue	Milk
[^{14}C]*Bicarbonate experiment*		
Casein	—	0·020±0·005
[1-^{14}C]*Hexanoate experiment*		
Casein	—	1·15±0·03
Whey proteins	—	0·23±0·03
Lactose	—	0·080±0·005
Volatile fatty acids	3·8±0·1	0·090±0·005
Non-volatile fatty acids	0·95±0·05	0·050±0·005
Phospholipids	0·57±0·05	0·00
Cholesterol	0·070±0·005	0·00
Glycerol	0·00	0·00

Chromatographic separation of fatty acids. The even-numbered straight-chain saturated and unsaturated fatty acids were fractionated chromatographically by the reversed-phase technique of Howard & Martin (1950) and by the vapour–liquid chromatography method of James & Martin (1952), as described previously. The fatty acids studied ranged from acetic acid to stearic acid.

Isolation of milk proteins. Isolation of milk proteins was carried out in both experiments as previously described. The incorporation of ^{14}C into amino acids of casein was measured after chromatographic separation (Hirs, Moore & Stein, 1954) as described previously.

Isolation of several Krebs-cycle acids from milk and udder tissue. Citric acid was isolated from milk in both experiments. Citric acid, fumaric acid and succinic acid were isolated from tissue in the hexanoate experiment only. The methods used for the isolation of citric acid and succinic acid have been described (Verbeke *et al.* 1959).

Fumaric acid was isolated as follows. 40 fractions, eluted from the Dowex-1 column after the citric acid peak, were combined and evaporated. The residue was dissolved in a minimal volume of 35% (v/v) butanol–chloroform and applied to the top of a silica gel column (Isherwood, 1946). Fumaric acid was eluted with 35% (v/v) butanol–chloroform, the emerging fractions being titrated with 0·01N-NaOH solution. Further purification of the fumaric acid peak fraction was carried out by paper chromatography (Kalbe, 1954). The acid solution was applied on the paper as a streak and the acid was localized after developing with methyl red indicator solution at pH 8·0. After elution with water, fumaric acid was estimated by the spot-area method.

Radioactivity measurements. The radioactivity measurements were made with a windowless flow counter as described previously (Verbeke *et al.* 1959). The magnitudes of the count rates obtained and the thicknesses of materials used are of the same order as described by Verbeke *et al.* (1959). In the tables the activities are recorded as μmc together with the S.D. from the mean from at least triplicate samples.

RESULTS

$NaH^{14}CO_3$ *experiment.* The casein isolated from milk in the bicarbonate experiment was labelled to only a slight degree as compared with that obtained in the hexanoate experiment (Table 1). As can be seen from Table 2, only glutamic acid and aspartic acid showed a measurable activity. In this experiment aspartic acid is slightly more active than glutamic acid.

Citric acid isolated from milk (Table 4) showed a very high specific activity as compared with aspartic acid and glutamic acid isolated from casein.

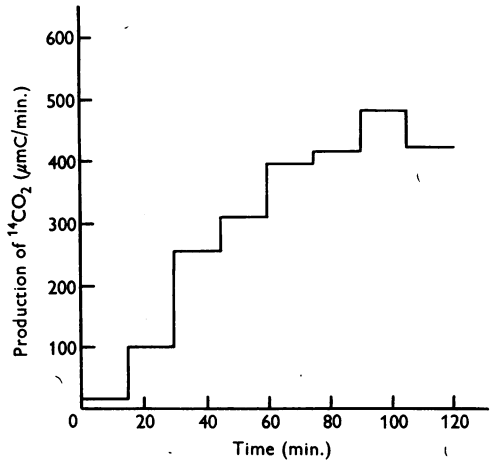

Fig. 1. Production of $^{14}CO_2$ during perfusion in the presence of [1-^{14}C]hexanoate.

Table 2. *Specific activities of amino acids isolated from casein after perfusion with* $NaH^{14}CO_3$ *or* [1-^{14}C]*hexanoate*

Specific activities are given as mμc/mg. of C (±S.D.).

Amino acid	Bicarbonate expt.	Hexanoate expt.
Glutamic acid	0·060±0·005	6·01±0·06
Aspartic acid	0·085±0·005	1·94±0·02
Serine	0·00	0·30±0·01
Alanine	0·00	0·050±0·005
Glycine	0·00	0·085±0·005
Proline	—	0·14±0·01
Valine	—	0·007±0·002

[1-^{14}C]*Hexanoate experiment.* Of the administered ^{14}C 3·6% was recovered as $^{14}CO_2$ liberated by the artificial-lung system. The method used enabled us to collect approx. 80% of the total $^{14}CO_2$. As can be seen from Fig. 1, $^{14}CO_2$ production increased progressively during the experiment to reach a maximum 90 min. after the start of perfusion.

Protein. The highest specific activities in milk were found in citric acid and casein (Tables 1 and 4).

The labelling of amino acids after addition of [1-^{14}C]hexanoate (Table 2) is similar to that obtained in previous perfusion experiments (Verbeke, Aqvist & Peeters, 1957; Lauryssens, Verbeke & Peeters, 1957; Verbeke *et al.* 1959) carried out in the presence of acetic acid, butyric acid and *iso*-valeric acid, showing that appreciable labelling of the non-essential amino acids has occurred. The essential amino acids isolated from casein showed no detectable level of radioactivity. In this experiment the highest specific activity observed was that of glutamic acid, this activity being three times that of aspartic acid.

Fatty acids. The specific activities of the fatty acids isolated from udder-tissue glycerides and milk glycerides are given in Table 3. The specific activity of the acids increases stepwise from butyric acid to octanoic acid and falls progressively from octanoic acid to stearic acid. Both udder and milk fatty acids give the same picture, udder fatty acids from butyric acid to palmitic acid being 25 to 75 times as active as the corresponding milk fatty acids.

The picture of the specific activities of the individual fatty acids is similar to the one obtained after perfusion in the presence of [1-^{14}C]butyric acid and [1-^{14}C]*iso*valeric acid.

About 14% of the ^{14}C dose administered was recovered in the glyceride fatty acid fraction.

Krebs-cycle acids. The specific activities of citric acid, succinic acid and fumaric acid were very high (Table 4). The specific activity of the citric acid fraction isolated from milk is higher than that of citric acid from tissue. The activity of succinic acid and fumaric acid fractions is definitely higher than that of citric acid. Similar results have been obtained recently in several perfusion experiments carried out in the presence of other ^{14}C-labelled precursors ([1-^{14}C]glucose and [6-^{14}C]glucose: unpublished work). It can be calculated that 1·5–2% of the ^{14}C dose added was recovered in citric acid.

Table 3. *Specific activities of even-numbered glyceride fatty acids from udder tissue and milk after perfusion with* [1-^{14}C]*hexanoate*

Specific activities are given as mμc/mg. of C (±S.D.).

Acid	Udder	Milk
Butyric	2·75±0·10	0·058±0·002
Hexanoic	5·10±0·12	0·057±0·003
Octanoic	9·52±0·10	0·132±0·003
Decanoic	8·75±0·10	0·119±0·003
Lauric	7·36±0·10	0·107±0·003
Myristic	1·75±0·06	0·094±0·004
Palmitic	1·45±0·03	0·050±0·002
Stearic	0·020±0·001	0·020±0·005
Palmitoleic [hexadec-7-enoic acid]	0·040±0·002	—
Oleic	0·070±0·003	0·010±0·003

Table 4. *Specific activities of some Krebs-cycle acids from milk and tissue after perfusion with* $NaH^{14}CO_3$ *or* [1-^{14}C]*hexanoate*

	Specific activity (mμc/mg. of C)
[^{14}C]*Bicarbonate experiment*	
Citric acid (milk)	1·12±0·01
[1-^{14}C]*Hexanoate experiment*	
Citric acid (milk)	25·7±0·03
Citric acid (tissue)	15·1±0·04
Succinic acid (tissue)	33·7±1·3
Fumaric acid (tissue)	71·2±1·5

DISCUSSION

The incorporation of sodium [^{14}C]bicarbonate into the amino acids of casein was very small. Only glutamic acid and aspartic acid showed a measurable specific activity. It can be calculated from the production of $^{14}CO_2$ in the presence of [1-^{14}C]-hexanoate that the carbonate pool was involved to only a very small extent in incorporation of ^{14}C into amino acids from [1-^{14}C]hexanoate. The same conclusion is applicable to previous perfusion experiments, in which the incorporation of other precursors, acetate, propionate (Verbeke *et al.* 1957), *iso*valerate (Verbeke *et al.* 1959) and butyrate (Lauryssens *et al.* 1957) into the amino acids of casein was studied. Milk citric acid, obtained from the bicarbonate experiment, showed considerable ^{14}C activity. However, the activity of this constituent in the hexanoate experiment was higher by a factor of 20. It appears that only a negligible fraction of the activity of citric acid in the hexanoate experiment can be attributed to $^{14}CO_2$ fixation. It was shown previously that $^{14}CO_2$ fixation into the fatty acid fractions and into lactose is negligible in the udder-perfusion experiment (Cowie *et al.* 1951). The same conclusion is applicable to casein and citric acid. $^{14}CO_2$ fixation into lactose and casein seems to be a much more important phenomenon in the intact dairy cow. A definite specific activity was noted by Black, Kleiber & Smith (1952) in many essential and non-essential amino acids of casein after intravenous injection of sodium [^{14}C]bicarbonate into lactating cows. It is obvious from these observations that ^{14}C activities in milk constituents are often easier to interpret in udder-perfusion experiments than in the investigations carried out on the intact cow.

Kleiber (1958) noted in the intact cow a considerable incorporation of [^{14}C]hexanoate and [^{14}C]butyrate into lactose of milk. Neither [1-^{14}C]-hexanoate nor [1-^{14}C]butyrate (Lauryssens *et al.* 1957) was acting in a glycogenic manner in the udder-perfusion experiment; in both the specific activity of lactose was very low.

It is evident that [1-^{14}C]hexanoate was broken down by the perfused udder into C_2 components with high acetylating capacity. These components are utilized as precursors of fatty acids and are metabolized by the Krebs cycle, yielding highly labelled citric acid, succinic acid and fumaric acid. The substantial specific activities detected in glutamic acid and aspartic acid from milk casein can easily be explained by transaminase activities. Small amounts of ^{14}C are presumably transferred by way of oxaloacetate to pyruvate, yielding lactose, alanine and serine with low activity. The same picture of ^{14}C incorporation was observed previously in the presence of acetate, butyrate and *iso*valerate.

If hexanoate and butyrate are acting as precursors of carbohydrates in the intact dairy cow but not in the perfused udder it may be supposed that another organ is involved in this conversion. Evidence that a reaction of this kind may occur in the liver of the cow was obtained recently by experiments on the perfused goat liver (Shaw, Lakshmanan, Chung, Leffel & Doetsch, 1958).

There is no evidence of direct esterification of [1-^{14}C]hexanoate, as seen from our results on the distribution of specific activities amongst the fatty acids. The analogy between the metabolism of butyrate and hexanoate in the perfused udder is very striking, both precursors being broken down to C_2 components.

The specific activities of the fatty acids from the udder tissue were 25 to 75 times as great as those found in milk, whereas in milk the casein fraction showed the highest activity. Similar results were obtained frequently in the presence of several precursors: [1-^{14}C]butyrate and [1-^{14}C]*iso*valerate. These results would suggest that hexanoate is acting primarily as a precursor of milk casein. However, this observation may be misleading as there are no data available on the specific activity of the casein in udder tissue. A more reasonable interpretation of these changes in specific activity is that they are a reflexion of the secretory processes in the udder cells. The maximum specific activity of the fatty acids in the intact cow is reached at a later time and the ^{14}C activity is maintained longer than in casein and lactose (Wood 1958).

SUMMARY

1. Perfusions of two halves of lactating cow udders were performed for 2 hr. with blood containing [^{14}C]bicarbonate and [1-^{14}C]hexanoate. Both halves received inactive acetate added continuously throughout the experiments, and 150 and 290 ml. of milk respectively were collected after perfusion.

2. A slight incorporation of $^{14}CO_2$ occurred into milk casein, largely in aspartic acid and glutamic acid. Citric acid isolated from the milk of the same half-udder showed a higher activity.

3. In the hexanoate experiment the specific activity of carbon dioxide was low at the beginning but increased gradually; approximately 4% of the ^{14}C was recovered as $^{14}CO_2$.

4. The specific activities of the milk constituents in the hexanoate experiment decreased in the following order: citric acid, casein, lower fatty acids, higher fatty acids and lactose. Cholesterol, glycerol and phospholipids did not show any detectable activity in milk. Fatty acid fractions isolated from udder tissue were approximately 40 times as active as the corresponding milk fatty acids.

5. The specific activity of the lower fatty acids from udder and milk increased with increasing chain length to reach a maximum at C_8 and then fell progressively with further increase of chain length. In the hexanoate experiment 14% of the added ^{14}C was recovered in fat at the end of the experiment. There was no evidence of direct esterification of [1-^{14}C]hexanoate.

6. Of the activity of milk casein obtained from the [1-^{14}C]hexanoate experiment 97% was due to glutamic acid and aspartic acid, the activity of the former being three times that of the latter.

7. These results are consistent with the assumption that [1-^{14}C]hexanoate is broken down to C_2 components with high acetylating capacity. These components are utilized for the synthesis of fatty acids and are metabolized by way of the Krebs cycle, which is demonstrated by the isolation of citric acid, succinic acid and fumaric acid with high specific activity. Hexanoate did not behave in a glycogenic manner in the perfused udder.

8. The carbonate pool is involved only to a negligible extent in the incorporation of ^{14}C from hexanoate into milk constituents by the perfused-udder preparation.

9. The specific activities of the different fractions may be a reflexion of the secretory processes of the udder cells. The fat formed in the cells is secreted in the alveoli at a later time than in the other milk constituents.

This work was supported by a grant of the Belgian I.R.S.I.A. Foundation. We thank Professor A. Van den Hende of the Agricultural College, Ghent, for the counting of radioactive samples.

REFERENCES

Black, A. L., Kleiber, M. & Smith, A. H. (1952). *J. biol. Chem.* **197**, 365.
Cowie, A. T., Duncombe, W. G., Folley, S. J., French, T. H., Glascock, R. F., Massart, L., Peeters, G. J. & Popják, G. (1951). *Biochem. J.* **49**, 610.
Gray, F. V., Pilgrim, A. F., Rodda, H. J. & Weller, R. A. (1952). *J. exp. Biol.* **29**, 57.
Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Isherwood, F. A. (1946). *Biochem. J.* **40**, 688.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
Kalbe, H. (1954). *Hoppe-Seyl. Z.* **297**, 19.
Kleiber, M. (1958). *2nd U.N. int. Conf. Peaceful Uses of Atomic Energy, Geneva*, paper 812.
Lauryssens, M., Verbeke, R. & Peeters, G. (1957). *Int. Conf. Radioisotop. Scient. Res., Paris*, paper 117.
McClymont, G. L. (1951). *Aust. J. agric. Res.* **2**, 92.
Peeters, G. & Massart, L. (1952). *Arch. int. Pharmacodyn.* **91**, 388.
Shaw, J. C., Lakshmanan, S., Chung, A. C., Leffel, E. C. & Doetsch, R. N. (1958). *2nd U.N. int. Conf. Peaceful Uses of Atomic Energy, Geneva*, paper 813.
Verbeke, R., Aqvist, S. & Peeters, G. (1957). *Arch. int. Physiol. Biochim.* **65**, 433.
Verbeke, R., Lauryssens, M., Peeters, G. & James, A. T. (1959). *Biochem. J.* **73**, 24.
Wood, H. G. (1958). *2nd U.N. int. Conf. Peaceful Uses of Atomic Energy, Geneva*, paper 852.

Parapepsins: Two Proteolytic Enzymes Associated with Porcine Pepsin

By A. P. RYLE* and R. R. PORTER
National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7

(*Received* 27 *February* 1959)

Several groups of workers have reported the presence of other proteolytic enzymes associated with pepsin, either in gastric juice or in extracts of the gastric mucosa. Freudenberg (1940), Pope & Stevens (1951), Buchs (1954) and Taylor (1959) have shown that activity may be found at pH values above 3·0 and this activity has generally been attributed to a cathepsin. These reports are discussed by Taylor (1959). Although Pope & Stevens (1951) were able to obtain a fraction showing a sharp pH optimum at pH 1·7 they were unable to purify a fraction with optimum in the range pH 3·0–4·0. Merten, Schramm, Grassmann & Hannig (1952), by precipitation and electrophoresis, and Richmond, Tang, Wolf, Trucco & Caputto (1958), by passage through a cation-exchange resin, obtained partial separations of the fractions with optimum activity below pH 2·0 and above pH 3·0.

Northrop (1932) obtained a fraction from pepsin which had a gelatin-liquefying activity much greater than that of pepsin.

We have fractionated crude pepsin by ion-exchange chromatography on diethylaminoethyl-cellulose and have isolated two minor components which differ from pepsin in specificity but are similar to it in other properties. One of these enzymes has also been found in a batch of crystalline pepsin.

This paper reports the isolation and characterization of these enzymes; a preliminary account of this work has already appeared (Ryle, 1958).

* Present address: Dept. of Biochemistry, The University, Edinburgh.

EXPERIMENTAL

Abbreviations used in this paper are as follows: acetyl-DL-phenylalanyl-L-di-iodotyrosine, APD; haemoglobin, Hb; dinitrophenyl, DNP; amino acid residues, those used by Brand & Edsall (1947).

Where the activities of the enzymes are expressed as m[P.U.] (defined below) without indication of the substrate, it is to be understood that APD was the substrate for parapepsin I and Hb for parapepsin II; for pepsin the units are equal for the two substrates.

Materials

The crude pepsin used was pepsin B.P. (sold by Hopkin and Williams Ltd., Chadwell Heath, Essex, and manufactured solely from pig gastric mucosae). Several different batches have been used, but no significant difference has been observed between them.

Diethylaminoethyl-cellulose was prepared according to Peterson & Sober (1956) and had about 1 m-equiv. basic groups/g.

Haemoglobin was bovine haemoglobin enzyme-substrate powder (Armour and Co. Ltd., Hampden Park, Eastbourne, Sussex), and was dialysed against water before use.

Acetyl-DL-phenylalanyl-L-di-iodotyrosine was kindly given by Mrs R. V. Pitt-Rivers of this Institute. This stereoisomeric mixture has not been crystallized, but was precipitated five times by acidification of an alkaline solution. It gave no reaction with ninhydrin.

Cysteyltyrosine and tryosylcysteine were also given by Mrs Pitt-Rivers.

Bovine plasma albumin was crystallized material obtained from Armour and Co. Ltd.

The milk powder was Gayelord-Hauser skim-milk powder, obtained locally from Boots Ltd.