Incorporation of DL-[1-¹⁴C]Leucine and [1-¹⁴C]isoValeric Acid into Milk Constituents by the Perfused Cow's Udder

By R. VERBEKE, MONIQUE LAURYSENS, G. PEETERS
Physiological Department of the Veterinary College, University of Ghent, Belgium

AND A. T. JAMES
National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 18 December 1958)

The older view on the composition of animal fats—that the only naturally occurring saturated fatty acids are those possessing an even number of carbon atoms—has in recent years been shown to be incorrect. Shorland and his co-workers in New Zealand (see Shorland, 1956) have in a series of papers demonstrated the occurrence in a number of fats of both branched and straight-chain odd-numbered acids. Similar results were obtained by James & Martin (1956) in the development of the gas-liquid chromatogram for the separation of the long-chain fatty acids, particularly large amounts of such branched and odd-numbered acids being found in the milk of ruminants.

The even-numbered acids have been shown to be synthesized in the udder of lactating animals by condensation of acetate units (Popják, French & Folley, 1951; Popják, French, Hunter & Martin, 1951; Cowie *et al.* 1951). The odd-numbered straight-chain acids, however, were found to be formed by the condensation of propionate with acetate in the isolated perfused cow's udder (James, Peeters & Lauryssens, 1956). The demonstration by El-Shazly (1952*a*) that the micro-organisms of sheep rumen were capable of deaminating amino acids to give the C₄ and C₅ branched-chain acids (*isobutyric acid*, *isovaleric acid* and *methylethyl-acetic acid*) suggested that some of these acids might be precursors of the long-chain *iso* and *anteiso* odd-numbered acids (El-Shazly, 1952*b*).

In order to investigate this point we have studied the utilization of both DL-[1-¹⁴C]leucine and [1-¹⁴C]*isovaleric acid* by the isolated perfused cow's udder, for synthesis of fatty acids, proteins and the Krebs-cycle acids.

EXPERIMENTAL

Udder perfusions. The two experiments were carried out on perfused lactating half-udders obtained from two different cows, by using the method described by Peeters & Massart (1952). Tests for chronic mastitis, with both clinical examination and the Hotis test (Hotis & Miller, 1936), were negative. Immediately before slaughter the cows were milked out with the aid of intravenously in-

jected oxytocin. The udders were removed and perfused for 2 min. with Ringer-Tyrode solution to remove blood, bisected along the median septum and connected to the perfusion apparatus. The teats were cut off at the top and cannulae were inserted; the residual milk was allowed to drain and was discarded. Perfusion was carried out with 8 l. of fresh heparinized cow's blood, the radioactive substrates being added to the artificial lung 30 min. after the perfusion was started. The following substrates were used: DL-[1-¹⁴C]leucine (0.95 mc, 23.37 mg.) in 50 ml. of water (after addition of a few drops of N-NaOH) and sodium [1-¹⁴C]*isovalerate* (0.5 mc, 62.06 mg.) in 50 ml. of water. After addition of the labelled materials, a solution of inactive sodium acetate (10.5 g. in 500 ml.) was added to the blood reservoir throughout the perfusion from a constant-drip device.

The gas evolved from the artificial lung was aspirated through NaOH solution to remove CO₂, the solution being replaced every 15 min. Approximately 80% of the evolved CO₂ was collected. The experiments were carried out under a large hood secured with a plastic curtain to prevent escape of labelled CO₂ into the atmosphere.

After 150 min. milk was released by injection of oxytocin (10 i.u.) into the udder artery, 160 ml. of milk being obtained in the leucine experiment and 140 ml. in the *isovalerate* experiment. The skin, teats and adipose tissue were removed by careful dissection and the residual udder tissue was cut into pieces and stored at -15° for 1 hr.

Extraction of fat. The milk fat was removed by centrifuging and dissolved in ether. The udder tissue was minced and extracted for several days with ethanol-ether (3:1, v/v) in a Soxhlet apparatus. The ethanol-ether was distilled off and the fat extracted with ether or light petroleum, insoluble material being discarded. From each kilogramme of wet tissue 30–50 g. of crude fat was obtained. Traces of labelled *isovaleric acid* were removed by washing the ether extracts several times first with 1% sodium bicarbonate and then with water. The ether layers were concentrated to small volume and the phospholipids were precipitated by adding acetone and a few drops of ethanolic MgCl₂. The neutral fat was saponified with ethanolic KOH on the water bath for several hours, water was added and the ethanol distilled off. Cholesterol was extracted with ether and precipitated as the digitonide. The aqueous layer was acidified with H₂SO₄ and the lower fatty acids were removed by steam-distillation; the long-chain acids were extracted with ether. Glycerol was extracted from the aqueous residue by refluxing with successive portions of ethyl acetate and precipitated as the trinitrobenzoate (Popják, Glascock & Folley, 1952).

Separations of the fatty acids. The steam-volatile fatty acid fractions (80–160 mg.) were dissolved in 35% (v/v) acetone in water and applied to the reversed-phase liquid-liquid column (Howard & Martin, 1950), containing 40 g. of Hyflo Super-Cel. The first zone eluted consisted of a mixture of acetic acid, butyric acid and caproic acid, the higher even-numbered acids being obtained as single peaks (C_8 – C_{14}). The sodium salts from the first zone were acidified and the free acids were extracted with ether and dried over anhydrous Na_2SO_4 . The ether was removed and the mixture of acetic acid, butyric acid and caproic acid was separated in 2 mg. amounts on the gas-liquid chromatogram (James & Martin, 1952).

The non-volatile long-chain fatty acid fraction (C_{14} – C_{18}) was separated initially on the reversed-phase liquid-liquid column. Each peak emerging was acidified, dissolved in ethyl acetate and hydrogenated over palladinized charcoal to a zero iodine value. Each peak was then rechromatographed. In this way all the even-numbered saturated and unsaturated acids from C_2 to C_{18} were separated. The resolving power of these liquid-liquid columns was insufficient to separate the odd-numbered straight- and branched-chain acids, so this was done with the gas-liquid chromatogram described by James & Martin (1956) and with the gas-density meter as detector (Martin & James, 1956). Fractions were collected as described by James *et al.* (1956).

Analytical methods. Total cholesterol was estimated according to Kenny (1952), iodine values by the semi-micro method of Trappe (1938) and glycerol by the Lambert & Neish (1950) technique.

Isolation of milk proteins. The skimmed milk was adjusted to pH 4.5, to precipitate the casein, which was redissolved in water, after centrifuging, by dropwise addition of N -NaOH to pH 7.0. This isoelectric precipitation and re-solution was repeated three times. Perchloric acid (6*N*) solution was added to the whey to give a final concentration of 0.35*N*. After standing overnight in the refrigerator the precipitate was removed by centrifuging and redissolved in water by addition of a few drops of N -NaOH. Addition of an equal volume of 20% trichloroacetic acid precipitated the whey proteins; these were washed three times with ethanol, then with ether and dried at 40°.

Amino acid separations. The casein samples were refluxed with 6*N*-HCl for 36 hr. Excess of HCl was removed by repeated evaporation *in vacuo*, the last traces being removed by leaving the hydrolysate overnight in a vacuum desiccator over soda lime. The residue was dissolved in water and the insoluble material deposited after being kept overnight at +2° was discarded. The pH was then adjusted to between 3.5 and 4.5 by dropwise addition of 0.1*N*-NaOH, and the solution was applied to a Dowex 1 column and eluted with 0.5*N*-acetic acid. In this way the acidic amino acids and tyrosine were separated, the neutral and basic amino acids composing the first zone eluted being resolved by a Dowex 50 column (Hirs, Moore & Stein, 1954). The mixed zones of serine and threonine and of isoleucine and methionine were separated on a starch column (Moore & Stein, 1949), being eluted with propan-1-ol-0.5*N*-HCl. All zones were located by the colorimetric procedure of Cocking & Yemm (1954), with a mixture of ninhydrin and KCN. Any contaminants introduced in the column procedures were removed by passing the amino acid solutions through small Dowex 50 columns, HCl being used for elution. The purity and identity of the separated amino

acids was checked by paper chromatography. The concentration of the amino acids in solution may be accurately determined by colorimetry, with the method of Cocking & Yemm (1954), or by the micro-Kjeldahl method with micro-diffusion techniques (Conway, 1950).

Isolation of some acids of the Krebs cycle. After extraction of casein and the whey proteins from the skimmed milk, the filtrate was adjusted to pH 3.0 with 5*N*-KOH. After storage at 2° for 24 hr. the precipitated $KClO_4$ was filtered off.

Udder tissue (500 g.) was homogenized at 2° in 2 vol. of iced water with addition of perchloric acid to a final concentration of 0.35*N*. The mixture was stored at 2° for 24 hr., the proteins were removed by centrifuging and resuspended in 0.35*N*-perchloric acid and centrifuged. The combined supernatants contained, in addition to the Krebs-cycle acids, a strongly acid viscous substance that interfered with the subsequent chromatographic procedures. This material was removed as follows: 5*N*-KOH was added to give a final pH of 3.0 and the mixture was stored at +2° for 24 hr. The precipitated $KClO_4$ was filtered off, the supernatant was concentrated *in vacuo* at 40°, filtered and passed through a Dowex 50 H^+ column. The acid solution emerging was treated with 10 vol. of ethanol and stored at 2° for several days; the resulting precipitate was filtered off, the filtrate neutralized with NaOH and evaporated to dryness *in vacuo*. The residue was dissolved in water and filtered.

Both milk and tissue extracts were passed through a Dowex 1 H^+ - CO_3^- column (30 cm. $\times$ 3 cm.) (Busch, Hurlbert & Potter, 1952). The column was washed with CO_2 -free water and gradient-elution was then done with formic acid ranging from 0 to 7*N*; 15 ml. fractions were collected. Lactic acid emerged as soon as the eluent became acid and was detected colorimetrically (Mendel & Goldscheider, 1925). Citric acid was detected (Natelson, Pincus & Lugovoy, 1948) in the fractions containing 2 to 3*N*-formic acid. The citric acid fractions were combined, evaporated, dissolved in a minimal volume of 35% (v/v) butanol- $CHCl_3$ and applied to a silica-gel column (Isherwood, 1946). The fractions eluted from the column were titrated with 0.1*N*-NaOH. In the analyses of the udder-tissue extracts, material eluted from the Dowex 1 column between lactic acid and citric acid was pooled and evaporated. The mixture was dissolved in a minimal volume of 35% (v/v) butanol- $CHCl_3$ and run on a silica-gel column. On elution with 10% (v/v) butanol- $CHCl_3$ two small peaks were produced containing succinic acid, malic acid and two unidentified acids. These substances were separated by paper chromatography with isoamyl formate-water-formic acid (11:1:2, by vol.), as described by Kalbe (1954). The mixture was applied as a streak and the zones were located with methyl red (pH 8.0); four well-defined zones were obtained, the fastest being unidentified, the second succinic acid, followed by another unidentified substance and then malic acid. The separated zones were eluted with water and further purified on a paper chromatogram with a phenol-xytol-85% formic acid (5:5:2, w/w/v) system. The zones were eluted with water, evaporated *in vacuo* over P_2O_5 and redissolved in 0.5 ml. of water. Malic acid and succinic acid were estimated on a one-dimension chromatogram by measurement of spot area with a planimeter, a series of standards of known concentration being used (Block, Durrum & Zweig, 1955). An accuracy of approximately 10% was obtained.

Radioactive assays. Most measurements were carried out with a windowless flow counter. The soaps were dissolved in a minimum amount of 50% ethanol and plated on plastic disks of 2 cm.², of the type described by Popják (1950). The samples were dried under an infrared lamp and weighed: usually amounts of 2–4 mg. were plated. An accurately known volume of the concentrated amino acid solution containing from 20 to 1000 μ g. of carbon was pipetted on the disks. Organic acids were dissolved in water and plated in the same manner. The dried samples were assayed for radioactivity. Lactose, glyceryl trinitrobenzoate, phospholipids, cholesterol digitonide and proteins were oxidized to CO₂ by the wet combustion method (Lindenbaum, Schubert & Armstrong, 1948) and the ¹⁴CO₂ was counted as BaCO₃. All counts were corrected for dead time of the instrument and background (32–39 counts/min.). Self-absorption corrections were made with the aid of experimentally determined curves (for soaps, amino acids, organic acids and BaCO₃). In the counting system used, 1 μ mc corresponded to 660 counts/min. above background. The limit which could be detected was 0.003 μ mc. The measurements of radioactivity were based on at least 6000 counts, giving a s.e. on the counting rate of 2% for samples with activity not less than 300 counts/min. (0.45 μ mc). In the tables we have recorded the activities as μ mc, together with the s.d. from the mean from at least triplicate samples. The long-chain fatty acid methyl esters were counted with an end-window counter as previously described (James *et al.* 1956).

RESULTS

Utilization of [1-¹⁴C]leucine

Carbon dioxide. The low catabolic activity of the leucine was demonstrated by the small amount of

Fig. 1. Activity (μ mc) of respiratory CO₂ after addition to the perfusion blood of —, [1-¹⁴C]isovaleric acid and ---, [1-¹⁴C]leucine.

¹⁴CO₂ liberated from the artificial lung. Only 1% of the added dose could be accounted for as CO₂. Production of the labelled CO₂ began slowly, increased to a maximum after 60–75 min. and then decreased up to the end of the experiment (see Fig. 1).

Protein. High activity was found in the milk proteins (Table 1). It can be calculated from the specific activities measured in the amino acids that most of the activity (99%) was due to leucine (Table 4). Low activity was detected in glycine and alanine and none in the other amino acids. The specific activity of the isolated casein was six times that found in the isovalerate experiment. The total activity of milk casein accounted only for 0.78% of the added ¹⁴C.

Table 1. *Specific activities of amino acids isolated from casein in the experiment with [1-¹⁴C]leucine*

For details see Experimental section.	
Isolated substance (milk)	Specific activity (μ mc/mg. of C)
Casein	5.10 $\pm$ 0.06
Whey proteins	4.73 $\pm$ 0.09
Leucine	52.0 $\pm$ 0.5
Glycine	0.080 $\pm$ 0.006
Alanine	0.010 $\pm$ 0.001
Serine	Trace
Valine	Trace

Table 2. *Specific activities of constituents of udder tissue and milk in the experiment with [1-¹⁴C]isovalerate*

For details see Experimental section.
n.d., not determined.

Substance	Specific activity (μ mc/mg. of C)	
	Udder tissue	Milk
Glyceride fatty acids		
Steam-volatile		
Water-soluble	2.30 $\pm$ 0.07	—
Water-insoluble	4.8 $\pm$ 0.2	0.020 $\pm$ 0.002
Non-volatile	0.75 $\pm$ 0.06	0.010 $\pm$ 0.002
Glycerol	0.00	0.00
Cholesterol	0.020 $\pm$ 0.001	n.d.
Phospholipids	0.55 $\pm$ 0.02	n.d.
Lactose	n.d.	0.050 $\pm$ 0.002
Casein	n.d.	0.87 $\pm$ 0.02

Table 3. *Specific activities of amino acids isolated from casein in the experiment with [1-¹⁴C]isovalerate*

For details see Experimental section. n.d., not determined.

Amino acid	Specific activity (μ mc/mg. of C)
Glutamic acid	4.42 $\pm$ 0.04
Aspartic acid	1.59 $\pm$ 0.02
Serine	0.190 $\pm$ 0.003
Glycine	0.038 $\pm$ 0.002
Alanine	0.030 $\pm$ 0.003
Proline	0.030 $\pm$ 0.003
Valine	0.018 $\pm$ 0.002

Table 4. Recovery of ^{14}C in the amino acids of total milk casein

Substance	[^{14}C]Leucine experiment		[^{14}C]isoValeric acid experiment	
	Total activity (μmc)	Percentage of total casein activity	Total activity (μmc)	Percentage of total casein activity
Casein	7818 $\pm$ 94	—	1013 $\pm$ 23	—
Glutamic acid	—	—	929 $\pm$ 9	89.3 $\pm$ 0.9
Aspartic acid	—	—	94 $\pm$ 1	9.0 $\pm$ 0.1
Serine	—	—	9.40 $\pm$ 0.15	0.90 $\pm$ 0.01
Glycine	2.10 $\pm$ 0.15	0.030 $\pm$ 0.002	0.80 $\pm$ 0.05	0.080 $\pm$ 0.005
Alanine	0.40 $\pm$ 0.04	0.0060 $\pm$ 0.0006	0.8 $\pm$ 0.1	0.080 $\pm$ 0.001
Proline	—	—	4.1 $\pm$ 0.4	0.390 $\pm$ 0.004
Valine	—	—	1.7 $\pm$ 0.2	0.160 $\pm$ 0.002
Leucine	7886 $\pm$ 79	99.9 $\pm$ 1.0	—	—

Fat. No activity could be detected either in the crude fatty acid mixtures obtained from the milk triglycerides or in the acids separated in the reversed phase column. The phospholipids and the lactose were also inactive.

Utilization of [^{14}C]isovalerate

Carbon dioxide. 5.5 % of the administered activity was recovered as CO_2 , compared with 1 % for leucine, although the time-activity curve had the same general shape; the maximum was reached at a later time (90 min.) (see Fig. 1).

Protein. Table 2 shows that casein is the most active of the protein, lactose and fat fractions isolated from milk. In this case labelling is found in all the non-essential amino acids, with glutamic acid and aspartic acid having the highest activity (Table 3). Little activity could be detected in serine, glycine, alanine and proline, and the essential amino acids were inactive. Of the added ^{14}C , 0.2 % was found in milk casein, aspartic acid and glutamic acid being responsible for 98 % of the labelling (Table 4).

Fatty acids. Much of the isovaleric acid is utilized in the synthesis of the triglyceride fatty acids, the udder-tissue acids having higher activities (by a factor of 100) than the milk acids. The specific activities of the individual acids are shown in Table 5. The specific activity of the lower fatty acids increases with increasing chain length, to reach a maximum at C_{10} , and falls progressively with further increase in chain length. Both udder and milk fatty acids give the same distribution pattern. Some quantitative analyses of the udder and milk lipids are given in Table 6, from which it can be seen that the two samples have, in general, similar compositions. In Table 7 the activities of the individual acids are expressed as a percentage of total fat activity. Separation of the branched- and straight-chain odd- and even-numbered acids by gas-liquid chromatography allowed a determination of their relative specific activities (see

Table 5. Specific activities of even-numbered acids from udder tissue and milk in the experiment with [^{14}C]isovalerate

Acid	Specific activity ($\mu\text{mc}/\text{mg. of C}$)	
	Udder tissue	Milk
Butyric	2.05 $\pm$ 0.03	0.00
Caproic (hexanoic)	2.83 $\pm$ 0.05	0.00
Caprylic (octanoic)	5.41 $\pm$ 0.05	0.06 $\pm$ 0.01
Capric (decanoic)	9.42 $\pm$ 0.09	0.070 $\pm$ 0.004
Lauric	8.49 $\pm$ 0.08	0.06 $\pm$ 0.01
Myristic	4.41 $\pm$ 0.08	(Lost)
Palmitic	1.08 $\pm$ 0.02	0.020 $\pm$ 0.001
Stearic	0.030 $\pm$ 0.005	0.00
Palmitoleic	0.030 $\pm$ 0.002	0.00
Oleic	0.040 $\pm$ 0.002	0.00
Citric	13.7 $\pm$ 0.1	16.8 $\pm$ 0.1
Succinic	38.2 $\pm$ 3.8	n.d.
Malic	26.9 $\pm$ 2.7	n.d.

n.d., not determined.

Table 6. Analytical characteristics of udder-tissue fat and milk fat in [^{14}C]isovaleric acid experiment

	Specific activity ($\mu\text{mc}/\text{mg. of C}$)	
	Udder tissue	Milk
Glyceride	89.0	88.6
Phospholipid	7.0	11.2
Total cholesterol	3.9	0.2

n.d., not determined.

Distribution of fatty acids in glycerides as molar %

Fatty acid	Udder tissue (g./100 g. of fat)	Milk (g./100 g. of fat)
Butyric	7.4 $\pm$ 0.4	9.2 $\pm$ 0.5
Caproic	2.3 $\pm$ 0.2	3.3 $\pm$ 0.2
Caprylic	1.5 $\pm$ 0.1	1.00 $\pm$ 0.05
Capric	5.6 $\pm$ 0.3	1.30 $\pm$ 0.06
Lauric	3.7 $\pm$ 0.2	0.60 $\pm$ 0.03
Myristic	7.5 $\pm$ 0.4	12.5 $\pm$ 0.6
Palmitoleic	1.9 $\pm$ 0.1	
Linoleic	3.1 $\pm$ 0.2	
Palmitic	28 $\pm$ 1	19 $\pm$ 1
Stearic	11.4 $\pm$ 0.6	9.9 $\pm$ 0.5
C_{14} unsaturated	0.50 $\pm$ 0.03	n.d.
Oleic	27 $\pm$ 1	38 $\pm$ 2
Highly unsaturated acids	0.40 $\pm$ 0.02	4.8 $\pm$ 0.3

Table 7. Recovery of ^{14}C in even-numbered fatty acid fractions in the experiment with $[1-^{14}\text{C}]\text{isovalerate}$

^{14}C recovered is expressed as percentage of total fat activities. —, no measurable activity.

Fatty acid	Udder tissue (%)	Milk (%)
Butyric	3.0 ± 0.2	—
Caproic	1.9 ± 0.1	—
Caprylic	3.2 ± 0.3	6 ± 1
Capric	26 ± 2	12 ± 1
Lauric	18 ± 1	5 ± 1
Myristic	23 ± 2	—
Palmitic	24 ± 2	77 ± 8
Stearic	0.30 ± 0.07	—
Palmitoleic	0.050 ± 0.006	—
Oleic	1.0 ± 0.1	—

Table 8. Specific activities of the long-chain acids from udder tissue

*iso*Valeric acid was used as precursor.

Acid	Counts/min./mg.
Lauric	491
Myristic	519
Branched pentadecanoic	598
<i>n</i> -Pentadecanoic	95
Palmitic	104
Branched heptadecanoic	110
<i>n</i> -Heptadecanoic	52
Stearic	5.6

Table 8). Approximately 20 % of the added ^{14}C was recovered in the various triglyceride fractions but little or no activity could be found in the isolated cholesterol.

The glycerides isolated from udder tissue were contaminated with cholesterol esters but determinations of free and total cholesterol showed that cholesterol esters were a minor component only.

Only a trace of activity could be detected in lactose.

Krebs-cycle acids. The specific activities of the isolated citric acid, succinic acid and malic acid were very high (Table 5), approximately 1.5 % of the added ^{14}C being present in citric acid. Unlike the fatty acid samples, the citric acid samples isolated from both udder tissue and milk had very similar specific activities.

DISCUSSION

The results reported demonstrate that although leucine is rapidly incorporated as such into milk proteins, especially casein, it acts neither as a precursor of fatty acids nor as an energy-yielding substrate.

*iso*Valeric acid, however, is readily metabolized and acts as a precursor of both fatty acids and certain of the non-essential amino acids. Its catabolic rate is about five times as great as that of leucine.

The specific activities of the fatty acids from the udder tissue were about 100 times as great as those of the acids found in milk. A similar effect was attributed by Cowie *et al.* (1951) to dilution of the secreted milk by residual milk present in the tissue at the beginning of the experiment. However, in the experiments reported here, citric acid from milk had a higher activity than that from udder tissue and, in an earlier experiment (Laurysens, Peeters, Coussens & De Loose, 1957), lactose samples isolated from milk and udder tissue did not have the widely different specific activities found frequently for fatty acids from the two sources. These results cannot be explained by any dilution effect but must represent different rates of secretion of the milk constituents.

The labelling of the even-numbered fatty acids found in the udder as well as that of the Krebs-cycle acids can only be due to breakdown of the carboxyl-labelled *isovaleric* acid between C-2 and C-3 to give a reactive C_2 unit, presumably acetyl-coenzyme A. This agrees with the scheme of *isovalerate* catabolism proposed by Greenberg (1954). The relative labelling of the different fatty acids is similar to that obtained from acetate (Popják, French, Hunter & Martin, 1951) in the living goat and from butyrate (Laurysens, Verbeke & Peeters, 1957) in the perfused udder.

This breakdown of the *isovalerate* must be extremely rapid for, despite attempts to slow degradation reactions by continuous addition of acetate, no selective incorporation of *isovalerate* into the branched-chain acids could be demonstrated. Indeed the radioactivity of these branched-chain acids can almost wholly be accounted for by the incorporation of acetate alone into the molecule. The synthesis of similar branched-chain acids such as β -hydroxy- β -methylglutarate, β -hydroxy*isovalerate* and $\beta\beta$ -dimethylacrylate from precursors such as acetate has already been demonstrated (see Rudney, 1957). Clearly then, the presence of branched-chain saturated acids in milk cannot be due to chain lengthening of these branched-chain precursors known to be present in blood but is caused by net synthesis within the mammary gland from a substance such as acetate.

The high specific activity of the casein amino acids, glutamic acid and aspartic acid, and the lower activity of glycine, serine, alanine and lactose, can be explained by the active C_2 fragment from *isovalerate* entering the Krebs cycle (see Turner, 1951) and being further converted into oxaloacetate and pyruvate. Similar relative labelling of these compounds was found in perfusion experiments with $[^{14}\text{C}]\text{acetate}$ and $[^{14}\text{C}]\text{butyrate}$ (Verbeke, Aqvist & Peeters, 1957; Laurysens, Verbeke & Peeters, 1957). The Krebs cycle in the mammary

gland therefore acts not only as an energy producer but also as a provider of the non-essential amino acids of milk protein.

SUMMARY

1. Two separated halves of lactating cow udders were perfused with blood containing respectively carboxyl-labelled leucine (0.95 mc of DL-[1-¹⁴C]-leucine, the 'leucine half') and carboxyl-labelled isovalerate (0.5 mc of [1-¹⁴C]isovalerate, the 'isovalerate half') for 2 hr. Both halves had inactive acetate added continuously throughout the perfusion.

2. The total quantity of ¹⁴C recovered as carbon dioxide was 1% in the 'leucine half' and 6% in the 'isovalerate half'.

3. [1-¹⁴C]Leucine was incorporated to a large extent into milk protein.

4. In the 'isovalerate half', ¹⁴C was incorporated into fatty acids isolated from the milk and udder glycerides. The specific activity of the lower fatty acids increased with increasing chain length, to reach a maximum at C₁₀, and fell progressively with further increase in chain length. No specific incorporation of ¹⁴C into branched-chain fatty acids could be demonstrated. Of the added ¹⁴C, 20% was found in the fat.

5. In the 'isovalerate half', casein was the most active of the milk constituents isolated. Of the ¹⁴C activity in this fraction, 98% was due to glutamic acid and aspartic acid. The isolated lactose was slightly radioactive.

6. These results are consistent with the assumption that [1-¹⁴C]isovaleric acid is broken down between C₂ and C₃, yielding a ¹⁴CO₂H-labelled C₂ component with high acetylating capacity. This component is metabolized by way of the Krebs cycle as shown by the isolation of very highly labelled citric acid, malic acid and succinic acid from the udder tissue.

7. In all the perfusion experiments carried out in presence of ¹⁴C-labelled precursors, the specific activities of udder-fat fractions were higher than those of the corresponding milk fractions by a factor varying between 10 and 100. The difference in specific activity of citric acid in udder and in milk is relatively very small.

8. The significance of these findings in relation to milk secretion is discussed.

Part of the expenses of this work were paid by a grant from the I.R.S.I.A. Foundation (R.V., M.L. and G.P.). Thanks are due to Misses J. Webb, A. Donck and M.T. Reinards and to Mr H. Hadaway for skilled help with part of this work.

REFERENCES

- Block, R. F., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Paper Electrophoresis*, p. 56. New York: Academic Press Inc.
- Busch, H., Hurlbert, R. H. & Potter, V. R. (1952). *J. biol. Chem.* **196**, 717.
- Cocking, E. C. & Yemm, E. W. (1954). *Biochem. J.* **58**, xii.
- Conway, E. J. (1950). *Microdiffusion and Volumetric Error*, p. 142. London: Crosby, Lockwood and Son.
- 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.
- El-Shazly, K. (1952a). *Biochem. J.* **51**, 640.
- El-Shazly, K. (1952b). *Biochem. J.* **51**, 647.
- Greenberg, D. M. (1954). *Chemical Pathways of Metabolism*, vol. 2, p. 66. New York: Academic Press Inc.
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.
- Hotis, R. P. & Miller, W. T. (1936). *Tech. Bull. U.S. Dep. Agric.*, no. 400.
- 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.
- James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.
- James, A. T., Peeters, G. & Laurysens, M. (1956). *Biochem. J.* **64**, 726.
- Kalbe, H. (1954). *Hoppe-Seyl. Z.* **297**, 19.
- Kenny, A. P. (1952). *Biochem. J.* **52**, 611.
- Lambert, M. & Neish, A. C. (1950). *Canad. J. Res.* **28**, 83.
- Laurysens, M., Peeters, G., Coussens, R. & De Loose, R. (1957). *Arch. int. Pharmacodyn.* **109**, 203.
- Laurysens, M., Verbeke, R. & Peeters, G. (1957). *Comm. int. Conf. Radioisotopes Sci. Res. Paris*.
- Lindenbaum, A., Schubert, J. & Armstrong, W. D. (1948). *Analyt. Chem.* **20**, 1120.
- Martin, A. J. P. & James, A. T. (1956). *Biochem. J.* **63**, 138.
- Mendel, B. & Goldscheider, I. (1925). *Biochem. Z.* **164**, 163.
- Moore, S. & Stein, W. H. (1949). *J. biol. Chem.* **178**, 53.
- Natelson, S., Pincus, J. & Lugovoy, J. (1948). *J. biol. Chem.* **175**, 745.
- Peeters, G. & Massart, L. (1952). *Arch. int. Pharmacodyn.* **91**, 388.
- Popják, G. (1950). *Biochem. J.* **46**, 560.
- Popják, G., French, T. H. & Folley, S. J. (1951). *Biochem. J.* **48**, 411.
- Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P. (1951). *Biochem. J.* **48**, 612.
- Popják, G., Glascock, R. F. & Folley, S. J. (1952). *Biochem. J.* **52**, 472.
- Rudney, H. (1957). *J. biol. Chem.* **227**, 363.
- Shorland, F. B. (1956). *Annu. Rev. Biochem.* **25**, 101.
- Terner, C. (1951). *Biochem. J.* **50**, 145.
- Trappe, W. (1938). *Biochem. Z.* **296**, 180.
- Verbeke, R., Aqvist, S. & Peeters, G. (1957). *Arch. int. Physiol. Biochim.* **65**, 433.