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Aspects of the metabolism of ^{14}C-labelled compounds by cows on a protein-free feed with urea and ammonium salts as the sole source of nitrogen*)

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Abstract. In the feeding experiments performed at the Biochemical Research Institute with test cows (the so-called 0-cows) the biosynthesis of milk component from different energy sources and from urea used as the nitrogen source was studied. The basic idea was to elucidate the effect of various feed components and substances formed in rumen fermentation on the biosynthesis of milk components. In the studies preparations labelled with ^{15}N and ^{14}C were used.

The feed of the test cows did not contain protein at all, the carbohydrates were hexose-based and fat in the form of oil was used very scantily. All the proteins required were synthesised by test cows in symbiosis with their rumen microbes from ammonium nitrogen which they obtained from urea and ammonium salts. Protozoa disappeared gradually from the rumen and the number of bacteria increased, becoming many tens of times the number in normally-fed cows.

Of the substances labelled with ^{14}C, stearic acid and acetic acid had the highest incorporation into the different milk components. Stearic acid is transferred to milk fat almost solely as such, but apparently is used for the formation of oleic, linoleic and linolenic acids as well. Acetic acid also is incorporated mainly into fat, though it is transferred in considerable amounts also to the other milk components.

Propionic acid is by nature gluconeogenetic and butyric acid lipogenic. The carbon of sucrose and lactic acid is incorporated fairly evenly into the various milk components.

The studies suggest that there are only very small amounts of aromatic compounds in 0-cow tissues.

According to the relative retention times the components of milk are synthesised from the different energy sources at various rates. The syntheses of citric acid and lactose are the most rapid, those of protein and fat the slowest.

The feeding has a marked effect on the composition of the milk fat. On the basis of these experiments, the far-developed urea feeding does not seem to have any pronounced effect on the participation of the substances studied in the biosynthesis of milk.

Introduction

Professor A. I. Virtanen started to study the synthesis of milk proteins at the Biochemical Research Institute as early as 1958. A cow on normal feed was then fed ammonium sulphate labelled with a heavy nitrogen atom

*) Lecture given at the Eastern Regional Research Center of the USDA, Philadelphia, 1977 and in honour of the 75th anniversary of the Hungarian Dairy Research Institute, Budapest, 1978.

(^{15}N) (LAND and VIRTANEN 1959, VIRTANEN and LAND 1959). As ^{14}N and ^{15}N react chemically in the same way and they can be determined with a mass-spectrometer very accurately, it was possible to follow in this test the participation of ammonium nitrogen in the synthesis of amino acids. The results showed that the amino acids of the milk proteins were labelled with ^{15}N already a few hours after feeding. The labelling of different amino acids was not, however, as rapid. Histidine and tryptophan had the slowest labelling. This observation led to studies on increasing the rumen microbial protein production of cows. Further, the formation of the aroma compounds of milk, besides protein synthesis, was a question to be elucidated by studies on production of milk of test cows, the so-called 0-cows fed purified, protein-free nutrients (VIRTANEN and LAMPILA 1962, VIRTANEN 1966, 1971, VIRTANEN et al. 1972).

In the same way as the synthesis of the amino acids of milk proteins from ammonia was followed by using urea and ammonium salts labelled with nitrogen-15, the synthesis of the carbon framework of protein, fat, lactose and citric acid from different energy sources by using preparations labelled with ^{14}C was followed. The basic idea of the ^{14}C studies was to elucidate the effect of various feed components and rumen fermentation products on the composition and amount of milk under closely controlled feeding conditions.

Feeding

The feed of 0-cows (0-feed) comprised purified carbohydrates, that is α-cellulose, starch, sucrose and glucose, and urea, ammonium salts, minerals, vitamins A, D and E, and vegetable oils. These components were fed in the form of pellets (Table 1). In addition to the pellets, the cows were fed moist cellulose paste. Also cellulose strips, impregnated at need with a solution of urea and glucose, were included in the feed. As fat the cows received daily 100—140 ml vegetable oil (mostly a mixture of maize and soybean oil). A daily amount of 100 000—200 000 IU of vitamin A and 20 000 IU of $D_2 + D_3$ was included in the feed. Since 1965 various amounts of DL -α-tocopherol were given to the cows.

It is of importance to note that, in addition to the absence of protein, the energy source in the feed of 0-cows is based on hexose sugars.

The first cow was included in the experiments in 1961. The adaptation of the cows to the test feed took place slowly. The portions of normal feed

Table 1. Composition (%) of the pellets in the feed of 0-cows.

	% *Range*
α-Cellulose powder	8.1 — 8.4
Potato starch	44.3 — 48.4
Sucrose	17.8 — 19.6
Mineral salt mixture	7.0 — 7.8
Urea + ammonium salts (Ratio 94: 6, calc. as urea)	3.7 — 5.4
Water	15.0

were decreased gradually and the test feed increased correspondingly. At the end of the lactation period the adaptation time was 1—2 months.

During the adaptation and test feed the microbial growth of the rumen of the test cows changed essentially. The number of bacteria in one milliliter of rumen contents rose to a figure 50—100 times higher than with cows on normal feed, and the protozoa disappeared either totally or their number was reduced so as to become insignificant.

The total amounts of volatile fatty acids in the rumen of 0-cows increased and the relative molar proportions changed when compared with cows on urea-rich, low-protein feed (ULP-cows) and on normal feed (NorP-cows) (Fig. 1).

During the first years the amount of urea fed corresponded to 16—18 g nitrogen per kg organic matter, the digestibility of which was 75—80 %. Since 1965 the amount of nitrogen fed was 23—28 g/kg organic matter. The increasing of the amount of urea had a vigorous influence on milk production. Fig. 2 shows 0-cow Oona, whose best annual milk production was 4 560 kg calculated as standard milk. Standard milk corresponds to 684 Kcal, that is 2.68 MJ, per kg milk.

In 1966 Professor Virtanen started also new feeding experiments with cows fed small amounts of protein in which the protein deficiency was corrected with urea, while hemicellulose and 0-fibre, a waste product of the cellulose industry, were included in the energy sources. This feed was called in our laboratory ULP-feed, and the cows ULP-cows, etc. An annual milk production

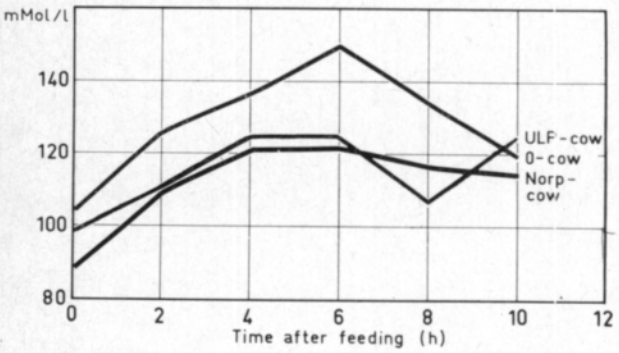

Fig. 1. Total volatile fatty acid content in the rumen of 0-, ULP- and NorP-cows before feeding and 2, 4, 6, 8 and 10 hours after feeding.

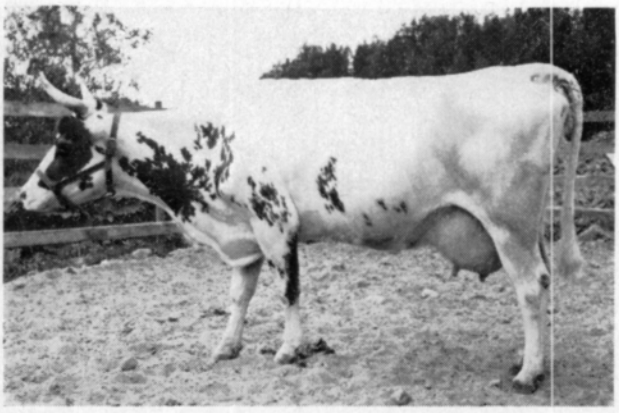

Fig. 2. 0-cow Oona, which had been 8 years on 0-feed and calved 6 times during this period, had a maximum yield of 4 564 kg milk per year calculated as standard milk. (684 Kcal, i.e. 2.68 MJ/kg milk).

of 5 000—6 000 kg was obtained on ULP-feed (VIRTANEN 1967, 1971, 1972, ETTALA and KREULA 1976).

Besides adding urea to the 0-feed, the effect of vitamins, individual amino acids, blood cells, ethanol extractables of potato, mineral mixtures and various oils and amounts of oil on the state of health, milk production and tissue composition of 0-cows was studed.

Total transfer of ^{14}C to milk, faeces and urine from ^{14}C-labelled compounds

Milk

According to usual milk analyses 0-milk contains 3.5—5.6 % fat, 3.5—4.0 % protein and 4.2—4.7 % lactose. Thus the contents of the components of 0-milk do not essentially differ from those of milk produced on normal feed (Table 2).

When following the synthesis of the carbon framework of milk protein, fat and lactose from different energy sources by using preparations labelled with ^{14}C, it was observed that the utilisation of carbon derived from various sources was different (Fig. 3). The largest amount of ^{14}C-labelled carbon was transferred to the milk components from stearic acid (42 %) and from acetate (32 %). The corresponding figures were from ethanol 16 %, propionate 14 %, xylose, n-butyrate, galactose and sucrose about 10 %, from cellulose less than 6 %, alanine 4 %, isobutyric acid 2.5 % and benzoic acid below 0.5 %.

Faeces

The largest amounts of activity in the faeces were derived from sucrose (16 %) and n-butyrate (16.2 %) (Fig. 4). The amounts of activity derived from alanine and glycerol recovered in the faeces were also great; the smallest amounts of activity were derived from ethanol, propionic acid and stearic acid.

Table 2. Composition of milk.

Protein	3.5 — 4.0 %
Protein composition	normal
Amino acid composition	normal
Enzyme activities	normal
Lactose	4.2— 4.7 %
Fat	3.5 — 5.6 %
Fat globule size distribution	normal
Creaming	abnormal
Fatty acid composition	abnormal
Iodine number	normal
Colour of fat	white
Vitamins	
Pantothenic acid	2 × normal
Biotin	5 × normal
Other vitamins	normal
Trace elements	normal
Flavour	normal
Freezing point depression	normal

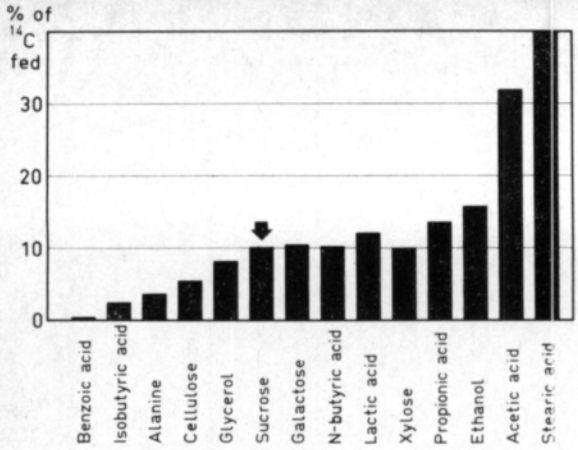

Fig. 3. Incorporation of ^{14}C into 0-milk.

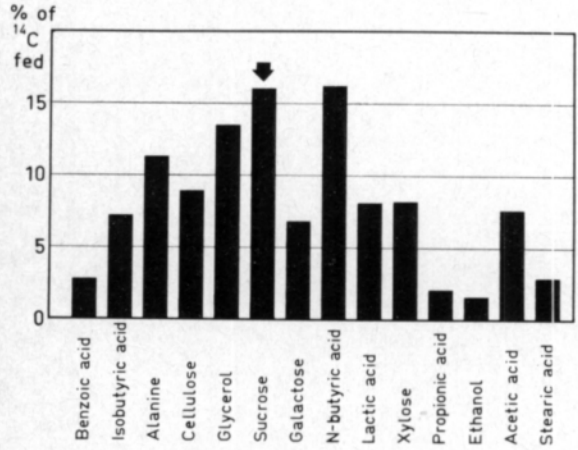

Fig. 4. Excretion of ^{14}C in the faeces of 0-cows.

The radioactivity recovered in the faeces is derived either from a radioactive carbon-containing substance used in the biosynthesis of undecomposed components of the rumen, or from excretions from the organism into the alimentary canal, such as digestive enzymes, bile acids etc.

Urine

The radioactivity recovered in the urine is derived from the metabolic residues of the organism (Fig. 5). This is clearly seen as regards benzoic acid and n-butyric acid.

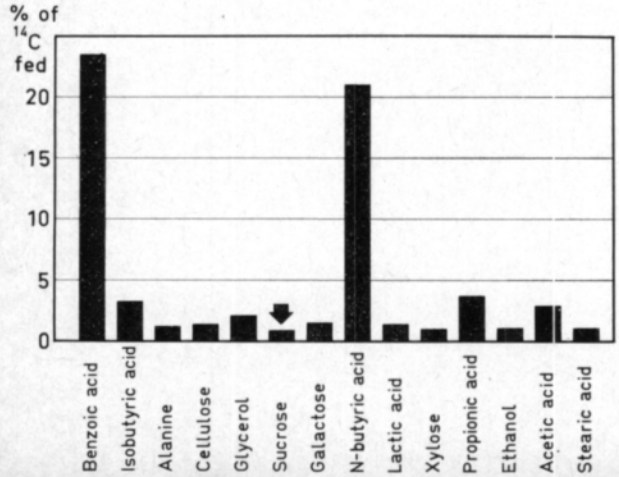

Fig. 5. Excretion of ^{14}C in the urine of 0-cows.

Total transfer of ^{14}C to milk components (lactose, protein, fat and citric acid)

Lactose

As regards the labelling of various milk components in different feeding experiments, the fact is that lactose is formed from glucose and galactose in the milk secretory cells of the mammary gland. Besides being obtained from the feed, glucose is also synthesised in the organism. The main substrates of this synthesis are propionic acid and proteins. When following the utilisation of labelled volatile fatty acids in the biosynthesis of milk components, it could be observed that very large amounts of propionic acid, even 9 % of the amount given, were transferred, *via* synthesis, to the lactose. On a starch-rich diet, however, the amount of glucose formed from propionic acid may remain smaller, since part of the starch may pass through the rumen, decompose and be absorbed from the lower parts of the alimentary canal. It is unlikely, however, that the slightly low lactose content of 0-milk is due to the lack of glucose. The glycogen contents of the liver of 0-cows have been clearly higher than those of normally-fed dairy cows and beef calves.

A significant detail to be mentioned is that in the biosynthesis of mik components from urea it is not only ammonia that is utilised but also carbon dioxide, which is formed when the urease enzyme decomposes urea into ammonia and carbon dioxide. Of the carbon of urea, as much as 3.7 % was found to be incorporated into milk components, mainly lactose. Also the carbon dioxide derived from formic acid was observed to partake in the biosynthesis of lactose. According to prevailing knowledge, the entry of carbon dioxide into gluconeogenesis is possible in such a way that it is bound in the formation of oxaloacetate. It is true that oxaloacetate is decarboxylated when phosphoenol pyruvic acid is formed form it. This is not, however, the same carbon dioxide which is bound to it; alternatively the ruminant may have other biosynthetic ways of reforming glucose.

Protein

The total protein content of 0-milk is slightly higher that that of normal milk. The results obtained when the milk proteins were fractionated by various methods show that the proteins of 0-milk and normal milk are very similar. Independent of feeding there are quantitative differences with regard to certain small protein fractions between different individual cows, but it seems that the differences are, at least in part, of a genetic nature. No differences were observed in the amino acid composition of the total protein and casein of milk compared with the corresponding amino acid composition of milk produced on normal feed

When studying the efficiency of the protein synthesis with a 0-cow fed urea labelled with ^{15}N, it could be observed that the labelling of the histidine and tryptophan of the milk proteins was almost double that of the normally-fed cows, but smaller than that of the other amino acids (Fig. 6). The biosynthesis of the carbon stems of the amino acids of milk proteins was also followed in the feeding experiments with ^{14}C-labelled ethanol and DL-lactate (Fig. 7). The results showed clearly the slight labelling of aromatic amino acids. Most

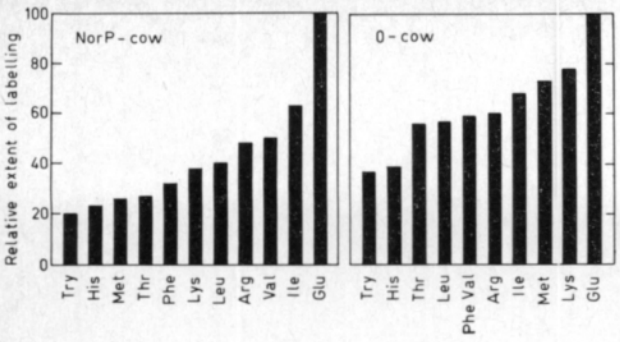

Fig. 6. Labelling of the amino acids of milk protein with a NorP- and a 0-cow after feeding ^{15}N-urea.

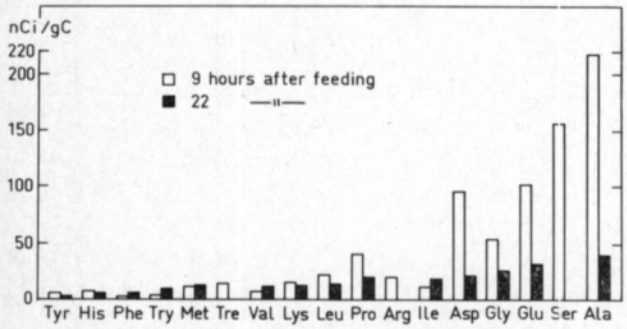

Fig. 7. Labelling of the amino acids of milk protein after feeding ^{14}C-lactic acid.

of the milk protein is labelled in the milk secretory cells of the mammary gland from their own free amino acids. The so-called essential amino acids are absorbed by the cells from the blood. Non-essential amino acids can be synthesised by the cells of the mammary gland, but even these are absorbed in part by the cells from the blood.

On 0-feed the content of the free amino acids in the blood plasma was in general smaller than on NorP-feed. The histidine content of the blood and particularly blood plasma was especially low during the highest milk production. Only the amount of glycine in the blood plasma of 0-cows was considerably higher than that of NorP-cows. Glycine is removed from the blood when it is conjugated with benzoic acid mainly in the liver. Benzoic acid is a metabolic product of aromatic compounds and also of aromatic amino acids, the detoxication of which takes place by conjugation with glycine to give hippuric acid. In the urine of 0-cows there are small amount of hippuric acid compared with the urine of ULP- and NorP-cows (Table 3). In the feeding experiment with benzoic acid uniformly labelled with ^{14}C, almost 99 % of the radioactivity fed was recovered in the urine of a ULP-cow, while the corresponding figure was only 26 % with a 0-cow.

The rumen microbes are indirectly involved in the synthesis of milk proteins. With 0-cows the rumen microbes synthesise amino acids and proteins by using urea and ammonium salts as the nitrogen source. When the microbial proteins are decomposed in the alimentary canal the amino acids are absorbed into the blood and in this way they are synthesised to milk protein. In the rumen of 0-cows the amounts of propionic and butyric acids in regard to acetic acid formed in the fermentation have increased compared with fermentations

Table 3. Hippuric acid contents in the urine (g/l) of 0-, ULP- and NorP-cows.

	Mean	No. of samples	Range
NorP-cows	11.9	14	5.3 – 23.5
ULP-cows			
Tila	3.1	14	0.2 – 7.8
Euru	7.0	42	1.1 – 12.8
0.cows			
Oona	0.6	66	0.1 – 1.9

on ordinary high-roughage feed. This increase of propionic and butyric acids is probably due to the fact that in the rumen of 0-cows the microbes ferment their solely hexose-based energy feed to a great extent also *via* the pentose-mono phosphate pathway. When moving from hexoses to pentoses, large amounts of reduced cofactors are formed; their re-oxidation involves a supply of propionic and butyric acids. The intermediary stages of the pentose-mono phosphate cycle are necessary in the biosynthesis of for example nucleic acids, histidine and aromatic amino acids. The re-oxidation of the reduced cofactors may become a factor regulating the whole fermentation procedure, and it is reflected also in the organism of the ruminant, leading for example to a deficiency of endogenic acetate, which can be seen in milk production and in its composition.

Fat

The greatest differences in the composition of the components of 0-milk are to be found when comparing the composition of the fat of 0-milk with that of NorP-milk. It is well known that the fatty acid composition of the feed has a great effect on the fatty acid composition of the milk of dairy cows. The palmitic acid content of the fat of 0-milk was particularly high, as much as 50 % of the total fatty acids, when the ration of the vegetable oil was 37 g per cow per day; at the time a total of 358—428 g fat was excreted in the milk. When the amount of oil was doubled, the palmitic acid content dropped to 40 % of the total fatty acids. When the amount of oil was at its smallest in the feed the amount of oleic acid in milk was low, only 10 % of the total fatty acids. When the amount of oil was raised to 129 g the amount of oleic acid of the total fatty acids was doubled, but it was still considerably lower than that of milk fat produced on normal feed. On different oil feeds the amount of stearic acid was low, rising to only half of that of normal milk.

Table 4. Relative specific activity of fatty acids of milk fat after administration of ^{14}C-labelled compounds. (Specific activity of milk fat = 100).

^{14}C test	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{18:0}$	$C_{18:1}$	$C_{1:82}$	$C_{18:3}$
Acetic acid	—	200	110	100	120	120	120	50	40	10	—
n-Butyric acid	—	—	150	140	150	180	140	50	15	16	15
Stearic acid	13	6	10	10	12	9	1	420	260	40	65
Xylose	180	160	120	120	110	120	110	25	35	10	8

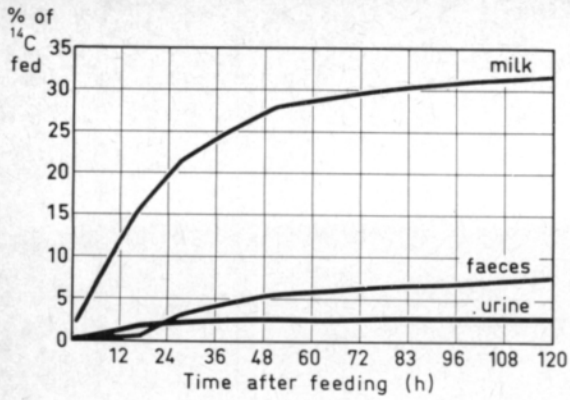

Fig. 8. Excretion of ^{14}C-activity in milk, faeces and urine in the acetic acid test with 0-cows.

In the feeding experiments with ^{14}C-labelled volatile fatty acids it was very obvious that acetic acid was used to a particularly great extent in the biosynthesis of all milk components. When ^{14}C-labelled acetate was fed to the cow, a total of 32 % of the ^{14}C was recovered in the milk, and of this amount about 3/4 was bound to milk fat (Fig.8). The milk fats of the first six milking portions were fractionated into their component fatty acids by means of preparative gas chromatography; the specific activity of each fatty acid (nCi/gC) was determined and its ratio to the specific activity of the original fat was calculated. It was observed that already in the first milking, two hours after the feeding of ^{14}C, it was divided rather evenly between all fatty acids except C_{18} fatty acids, and that this situation remained almost unchangeable for at least three days (Table 4). When ^{14}C-labelled stearic acid was fed, it was observed that of the amount of ^{14}C fed as much as 42 % was transferred to milk and almost all to milk fat.

In the acetate test the low activities of C_{18} fatty acids are due to the fact that these fatty acids are derived mostly from the inactive C_{18} fatty acids on the blood (Table 4). On the other hand the C_{16} ratio was higher than what was expected, for several workers have found earlier that a large proportion of the C_{16} fatty acid of milk fat is transferred as such from the blood to the milk. Apparently the 0-cow, in contrast to the NorP-cow, synthesises all, or almost all, of the C_{16} fatty acid of its milk in the mammary gland. In the feeding experiment with stearic acid the high ratio of $C_{18:0}$ fatty acid compared with the others shows the vigorous transfer of stearic acid as such from the blood to the milk secretory cells of the mammary gland and in this way to milk fat.

Citric acid

The citric acid content in the milk of 0-cows is normal, that is 0.2 %. The labelling of citric acid was very rapid and vigorous. Radioactivity derived from acetic acid in particular was transferred in large amounts to the citric acid in the milk (Fig. 9). This can be clearly established also by relative retention times (Table 5). The most rapid syntheses from the various energy sources were the syntheses of citric acid and lactose, the slowest those of protein and fat.

The results obtained in the ^{14}C studies under controlled feeding conditions show that the various feed components have a clear effect on the composition and amount of milk. The results are of significance also in the feeding of dairy cows.

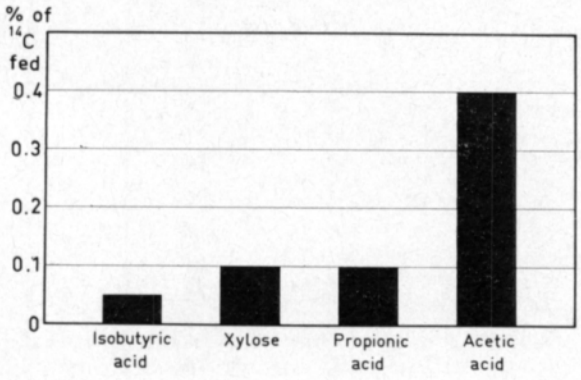

Fig. 9. Incorporation of ^{14}C into citric acid of 0-milk.

Table 5. Retention times of ^{14}C in whole milk and milk components of 0-cows.

^{14}C-test	Average Retention Time (h) in				
	Milk	Fat	Protein	Lactose	Citric acid
Acetic acid	31.2	32.5	29.1	25.0	18.8
Propionic acid	25.6	36.8	34.1	20.8	32.5
n-Butyric acid	34.9	39.8	38.9	24.7	—
Sucrose	20.3	22.3	23.7	14.6	—
Lactic acid	17.9	17.0	27.8	13.6	—

REFERENCES

Ettala, T. & Kreula, M. 1976. Milk production on low-protein, urea-rich feed. Acta Agr. Scand. 26: 33—39.

Land, H. & Virtanen, A. I. 1959. Ammonium salts as nitrogen source in the synthesis of protein by the ruminant. Acta Chem. Scand. 13: 489—496.

Virtanen, A. I. 1966. Milk production of cows on protein-free feed. Science 153: 1603—1614.

— 1967. Milk production on a protein-free and protein-poor feed. Neth. Milk & Dairy J. 21: 223—244.

— 1971. Protein requirements of dairy cattle — artificial nitrogen sources and milk production. Milchwissenschaft 26: 129—138.

— , Ettala, T. & Mäkinen, S. 1972. Milk production of cows on purified protein-free feed with urea and ammonium salts as the only nitrogen source and on non-purified feed with rising amounts of true protein. Festskr. 70-års dag Prof. Dr. Agr. h.c. K. Breirem 1972, p. 249—275.

— & Lampila, M. 1962. Production of cow's milk on purified nutrients without proteins. Suom. Kemistilehti B 35: 244.

— & Land, H. 1959. Synthesis of amino acids and proteins from ammonium salts by ruminants. Acta Agr. Fenn. 94: 1—7.

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Näkökohtia ^{14}C:llä leimattujen yhdisteiden metaboliasta proteiinittomilla, puhdistetuilla rehuilla ruokituilla koelehmillä

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Biokemiallisessa Tutkimuslaitoksessa on seurattu ns. 0-ruokintakokeiden yhteydessä lypsylehmillä maidon aineosien biosynteesiä. Tutkitut aineet ovat olleet joko luonnollisten rehujen aineosia tai pötsikäymisissä muodostuneita aineita. Käytetyt preparaatit ovat olleet joko ^{15}N:llä tai ^{14}C:llä leimattuja.

Koelehmien ruokintaan ei ole sisältynyt lainkaan proteiineja, hiilihydraatit ovat olleet heksoosipohjaisia ja rasvaa öljyn muodossa on käytetty niukasti. Kaiken tarvitsemansa valkuaisaineet koelehmät ovat syntetisoineet symbioosissa pötsimikrobiensa kanssa. Typenlähteenä on ollut urea ja ammoniumsuolat. Alkueläimet ovat joko kokonaan hävinneet pötsistä tai niiden lukumäärä on supistunut mitättömäksi.

^{14}C:llä leimatuista yhdisteistä steariinihappoa ja etikkahappoa on käytetty eniten maidon aineosien synteesissä. Steariinihappo on siirtynyt lähes sellaisenaan maitorasvaan, mutta sitä on käytetty pieniä määriä öljy- ja linolihapon sekä todennäköisesti myös linoleenihapon muodostukseen. Etikkahappoa on käytetty lähinnä maitorasvan, pääasiassa palmitiinihapon ja sitä lyhyempiketjuisten rasvahappojen synteesiin. Sitä on siirtynyt runsaasti myös maidon muiden aineosien muodostukseen. Propionihappo on luonteeltaan glukoneogeneettinen ja voihappo lipogeeninen. Sakkaroosin ja maitohapon hiiltä on käytetty melko tasaisesti maidon eri aineosien muodostukseen.

Virtsan hippuurihappopitoisuuksien perusteella näyttää aromaattisten yhdisteiden määrä olevan 0-lehmien elimistössä vähäinen ja ^{14}bentsoehappokokeen perusteella bentsoehapon detoksikaatio puutteellinen.

Eri yhdisteistä peräisin olevan radioaktiivisuuden keskimääräinen viipymä osoittaa maidon aineosien muodostuvan samasta hiilenlähteestä eri nopeuksilla. Nopeimmin näyttävät syntetisoituvan sitruunahappo ja laktoosi, hitaimmin valkuaisaineet ja rasva.