

3. In the same time nearly 35 % of the benzene is eliminated as metabolites in the urine. These consist mainly of conjugated phenols. Phenol is the major metabolite accounting for about 23 % of the dose. The other phenols excreted are quinol (4.8 %), catechol (2.2 %) and hydroxyquinol (0.3 %). *p*-Phenylmercapturic acid accounts for 0.5 % of the dose.

4. The only open-chain acid which is found in the urine and is derived from benzene, is *trans-trans*-muconic acid (1.3 % of the dose). Radioactive *cis-cis*- and *cis-trans*-muconic acids, acetic, oxalic, adipic, succinic, fumaric and mesotartaric acids were not found in the urine. The carbonate of the urine contained no radioactivity.

5. Diphenyl and *p*-diphenylglucuronide added to the radioactive urine were recovered devoid of activity, and hence these are not produced biologically from benzene.

6. The respiratory and urinary excretions account for nearly 80 % of the dose of benzene in 2-3 days after dosing, and the remainder appears to remain in the body to be eliminated slowly in the urine and probably in the expired air as carbon dioxide.

7. Two days after dosing, about 5 % of the administered radioactivity was found in the tissues generally. This occurred mainly as metabolites of benzene, and very little unchanged benzene was found after this time.

8. These experiments account for 80-90 % of the administered benzene.

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## Biosynthesis of Milk-fat in the Rabbit from Acetate and Glucose. The Mode of Conversion of Carbohydrate into Fat

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The main object of the investigation described in this article was to study the mode of conversion of carbohydrate into fat. It will be shown that glucose is converted very effectively not only to fatty acids but also to glycerol. With the aid of acetate, pyru-

vate and glucose labelled with <sup>14</sup>C it has been possible to demonstrate that the conversion of glucose into fatty acids takes place after the breakdown of glucose to acetate, probably via pyruvate. The lactating rabbit was used as the experimental animal and the biosynthesis of glyceride fat studied in the mammary gland, because recent experience has shown that the mamma of a lactating animal is particularly suited for such purpose (cf. reviews by Folley, 1952; Popják, 1952*a, b*).

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In addition to the primary aim of the investigation quantitative data have been obtained which define with some degree of probability the percentage of milk fatty acids (especially of short-chain fatty acids) derived from acetate and glucose respectively. Some of the results have already been communicated in a preliminary form (French & Popják, 1951; Popják, 1952*b*, *c*).

### EXPERIMENTAL

Rabbits (wt. 2.5 kg.) between the 12th and 16th days of lactation were used. Animals with a litter of four or more were preferred on account of their better yield of milk. The treatment of all experimental animals was the same unless stated otherwise. For 2–3 days before the experiment the mothers were separated from their litters in the morning and were milked 5–6 hr. later in order to make them accustomed to handling. For the night they were put back with the young.

For the actual experiment the mothers were again separated from the litters and, without preliminary milking, the test substance(s) administered. They were milked 6 hr. later and, when the experiment required, were killed by a dose of Nembutal and the mammary glands and the liver removed.

The comparison of acetate and carbohydrate as sources of milk fat was made in some experiments in the same animal. In such case [ $^{14}\text{C}$ ]acetate was given first and the animal milked 6 hr. afterwards. [ $^{14}\text{C}$ ]starch or [ $^{14}\text{C}$ ]glucose was administered 3–4 days later, when radioactivity was barely detectable any more in the milk fat, and the animal milked again 6 hr. afterwards. All animals were fed *ad lib.* before and during the experimental periods. This was essential in order not to interfere in any way with milk production and with the general metabolism of the animal. Ample evidence is now available to show that fasting fundamentally modifies lipid metabolism.

**Milking.** The animals were given intravenously 2–4 i.u. of oxytocin and were milked by applying intermittent suction of about 14 cm. of Hg to the nipples through the apparatus shown in Fig. 1. The funnel-shaped end of a male-urethral catheter served conveniently as the 'teat-cup'. The intermittent suction was made by closing and opening with the finger side arm *A* in Fig. 1. This apparatus proved far more flexible than an automatic milking machine, which was also tried, as it allowed a free variation of the rate of suction according to the 'let-down' of milk. The best yields of milk were obtained by applying intermittent suction at the rate of 30–40 times/min. at the beginning of milking and then, when the milk began to flow freely, by increasing the rate of suction to 80–100 times/min. During the periods of suction the 'teat-cup' was gently pulled so that not only suction, but also traction was applied to the nipples. The yield of milk varied a good deal from animal to animal; on the average about 25 ml. of milk could be obtained in less than half an hour, but some animals gave as little as 10 ml. or as much as 55 ml. The animals were held lightly on their backs by an assistant during milking; no restraint or anaesthetic was required.

**Substances administered.** These were: [*carboxy*- $^{14}\text{C}$ ]acetate and [*Me*- $^{14}\text{C}$ ]acetate (obtained from the Radiochemical Centre, Amersham, Bucks); [ $^{14}\text{C}$ ]starch and [ $^{14}\text{C}$ ]glucose

prepared from tobacco leaves which were allowed to photosynthesize in an atmosphere of  $^{14}\text{CO}_2$  (Porter & Martin, 1952). Samples of [ $1\text{-}^{14}\text{C}$ ]glucose were kindly given by Dr R. Bentley and Dr K. Bloch. [ $\alpha\text{-}^{14}\text{C}$ ]Pyruvamide and [ $\beta\text{-}^{14}\text{C}$ ]pyruvamide were synthesized as described by Anker (1948*a*); the pyruvate was freshly prepared before each experiment by the hydrolysis of pyruvamide with HCl followed by neutralization with NaOH. The labelled acetate and glucose were given in solutions, either by vein or by stomach tube and made isotonic with NaCl when necessary. The starch, in solution, was administered by stomach tube. Pyruvate was given always by vein.

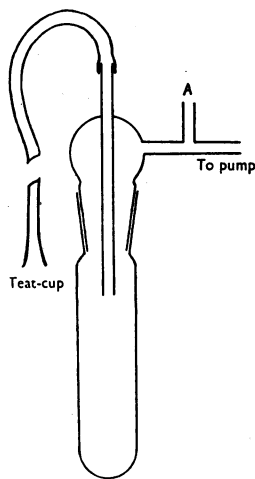


Fig. 1. Apparatus used for milking rabbits. Intermittent suction was applied to the nipples by closing and opening side arm *A* with the finger.

*p*-Aminobenzoic acid (1 g.) was dissolved in slight excess of *n*-NaOH, the pH adjusted to about 7 with HCl and the volume of the solution made up to 50 ml. with water. It was given by stomach tube.

**Extraction of fat from the milk, mammary gland and liver.** The milk was poured into 20 vol. of ethanol-ether mixture (3:1, v/v) and boiled for 5 min., the extract filtered hot and the residue re-extracted three times with 5 vol. of ethanol-ether. All extracts were combined.

The mammary gland, freed from connective-tissue fat as well as possible, was frozen with solid  $\text{CO}_2$  and crushed in a chilled mortar as it was found very difficult to divide the gland finely by other means. The liver was minced for the extraction. The extraction of the tissues and fractionation of lipids and fatty acids were done as described previously (Popják & Beeckmans, 1950; Popják, French & Folley, 1951). Octanoic acid for chemical degradation (see later) was isolated from the water-insoluble steam-volatile fatty acids of milk and mammary-gland fat by vacuum distillation in a Podbielniak Heligrad-packed microdistillation column (Podbielniak Inc., Chicago, Ill., U.S.A.).

**Preparation of lactose from milk.** The ethanol-ether extract of the milk was evaporated to dryness under reduced pressure and the fat was extracted with light petroleum (b.p. 40–60°). The light-petroleum extract usually contained some finely suspended material, most of which was lactose and was separated by centrifugation. The residue in the

distillation flask, after the extraction with light petroleum, was almost pure lactose (often crystalline) and was dissolved in about 5 ml. of distilled water and added to the small amount of lactose separated from the light petroleum. This crude lactose solution was shaken three times with 3 ml. of ether and centrifuged each time; the ether was pipetted off leaving a thin pad of insoluble material on the surface of the aqueous solution which was filtered after the last extraction with ether and concentrated to a syrup on a hot-water bath. Boiling ethanol was added to the hot syrup until faint cloudiness appeared in the solution. Lactose crystallized out at 4° usually in 24 hr.; it was purified by crystallization from 60% (v/v) ethanol, washed with absolute ethanol and acetone and dried *in vacuo*.

*Preparation of glycerol tribenzoate from glycerides.* The method employed has been described previously (Popják, Glascock & Polley, 1952).

*Extraction and degradation of p-acetamidobenzoic acid from urine.* The acidified urine was extracted with ether in a continuous extractor for 24 hr. as described by Bloch & Rittenberg (1945). The purification of *p*-acetamidobenzoic acid by crystallization from hot water after treatment with charcoal, as described by these and other authors, was found, however, to involve very large losses on account of the strong adsorption on charcoal. Purification through the sodium salt was found far more satisfactory. The ethereal extract of the urine was evaporated to dryness and there were added to the residue 10 ml. of *N*-HCl which dissolved any free *p*-aminobenzoic acid together with a great deal of yellow pigment. The crude *p*-acetamidobenzoic acid was filtered off on a small Büchner funnel, washed with cold water, suspended in 20 ml. of water and dissolved by adding 0.1 *N*-NaOH to about pH 8. The solution was made up to 80 ml. with distilled water and made acid to pH 4 with HCl, when a dark-brown flocculent precipitate separated out. This was centrifuged off leaving a slightly coloured solution of *p*-acetamidobenzoic acid which was extracted immediately into ether and the ether evaporated off. The same process as above was repeated twice more. After the third purification the volume of the alkaline solution was kept down to 10 ml.; after acidification with HCl to pH 3-4, *p*-acetamidobenzoic acid began to crystallize almost immediately. The crystallization was completed overnight in the cold. The crystals were filtered off, washed with ice-cold distilled water and dried at 110°; m.p. 254-256°. From the urine (100-150 ml.) collected for 6 hr. after the administration of 1 g. of *p*-aminobenzoic acid usually 200-300 mg. of *p*-acetamidobenzoic acid were obtained.

For the separation and chemical degradation of the acetyl group a weighed amount of *p*-acetamidobenzoic acid was hydrolysed with 30% excess of ethanolic KOH on a boiling-water bath for 3 hr.; the ethanol was evaporated off and gradually replaced by water. After acidification with H<sub>2</sub>SO<sub>4</sub> the acetic acid was separated by steam distillation. Usually 1 ml. of *N*-acetic acid was added to the mixture before distillation. The distillate was titrated with 0.1 *N*-LiOH and the solution evaporated to dryness. Silver acetate was prepared for radioactive counting from a small sample of the lithium acetate, the remainder being used for degradation by pyrolysis at 380-400° in a manner similar to that described for barium acetate by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). The degradation of labelled lithium acetate by pyrolysis gives an error in that 1.2% of the activity of the carboxyl carbon appears in the CHI<sub>3</sub> (derived from the methyl carbon of acetone, hence

from the methyl carbon of acetate), and 2% of the activity of the methyl carbon of acetate appears in the Li<sub>2</sub>CO<sub>3</sub> (carboxyl carbon) of acetate. The values of radioactive counts were corrected for these errors. The CO<sub>2</sub> from the Li<sub>2</sub>CO<sub>3</sub> was liberated with lactic acid *in vacuo*, trapped in Ba(OH)<sub>2</sub> and assayed for <sup>14</sup>C content as BaCO<sub>3</sub>. The CHI<sub>3</sub> was oxidized to CO<sub>2</sub> *in vacuo* by the technique of Van Slyke, Plazin & Weisiger (1951) and the CO<sub>2</sub> collected as BaCO<sub>3</sub>.

*Partial degradation of octanoic acid.* Carbon atoms 1 and 2 (carboxyl carbon and  $\alpha$ -carbon) were obtained as acetic acid and carbon atoms 3-8 as caproic acid by a method previously described (Hunter & Popják, 1951). The acetic acid was further degraded by pyrolysis of lithium acetate. Carbon atoms 7 and 8 were also obtained as acetic acid by chromic acid oxidation of octanoic acid (Kuhn & Roth, 1933). The acetic acid was obtained from the oxidation mixture by steam distillation and was further purified by partition chromatography (stationary phase 0.5 *N*-H<sub>2</sub>SO<sub>4</sub>, moving phase CHCl<sub>3</sub>; CHCl<sub>3</sub>-butanol (9:1, v/v)). It was found that the oxidation product was not pure acetic acid, but a mixture of acetic (75-80%) and of propionic acid (20-25%). Only the pure acetic acid was used for further degradation to obtain carbon atoms 7 and 8.

*Assay of <sup>14</sup>C.* This was done on solid samples of infinite thickness as previously described (Popják, 1950).

## RESULTS

### *Synthesis of fat in the mammary gland of lactating rabbits*

The synthesis of fatty acids and of cholesterol in the mammary gland of non-lactating pregnant rabbits, at least 1 week before parturition, has already been demonstrated. This was shown by the incorporation of deuterium from the body water into these substances at a rate greater than that which occurred in the liver lipids (Popják & Beekmans, 1950) and by the utilization of [<sup>14</sup>C]acetate for both fatty-acid and cholesterol synthesis in the mammary gland (Popják, Folley & French, 1949; Popják & Beekmans, 1950). It remained yet to be proved that this was also the case during lactation, although Folley & French (1950) have inferred from respiratory measurements made on tissue slices that the mamma of lactating rabbits also synthesizes fat from at least glucose.

It was known to us from other experiments on rabbits that the highest radioactivity in the plasma lipids occurred about 24 hr. after the injection of [<sup>14</sup>C]acetate. It was argued, therefore, that if the specific radioactivity of the fatty acids and of cholesterol in milk and mammary gland were to exceed the radioactivity of the fatty acids and of cholesterol in the liver (this organ being the source of plasma lipids) much before the time at which the maximum activity of the plasma lipids was observed, proof of the occurrence of synthesis in the mammary gland would be obtained. A time of 6 hr. after the administration of a particular labelled substance was chosen as a convenient experimental point.

It should be mentioned that the specific activities of the fractions of milk and mammary-gland fat were found equal 6 hr. after administration of either labelled acetate, pyruvate or glucose. For this reason and in order to simplify presentation of data only one set of figures will be given in the tables either under the heading of 'mammary-gland fat' or 'milk fat'. In the former case it is implied that the milk fat was analysed together with the mammary-gland fat, and in the latter that only the milk fat was studied: Lactose was, however, obtained always from the extraction of milk alone.

The data in Table 1 show that the specific activities of the substances extracted from the mammary gland were appreciably greater than those obtained from the liver 6 hr. after the administration of either [ $Me-^{14}C$ ]acetate or of [ $^{14}C$ ]glucose. The two experiments are typical of several others which yielded essentially similar results. Fatsynthesis from acetate and from carbohydrate in the mammary gland of lactating rabbits may therefore be taken as proved.

*Comparison of acetate and carbohydrate as precursors of milk fat*

It was thought necessary to make a comparison between acetate and carbohydrate as precursors of milk fat in the same experimental animal (cf. Experimental section) in order to determine whether or not either of these substances is used preferentially by the mammary gland. In Table 2 the results of two such experiments are recorded (see also Table 5) and show that the counting rates given by the fatty acids and by cholesterol were very similar after the administration of equal

radioactive doses of acetate and of carbohydrate, i.e. the results do not suggest a preferential utilization of acetate over carbohydrate or vice versa for fatty-acid and cholesterol synthesis. However, the radioactivities of lactose and of glycerol indicate, as will be discussed later, that glucose acts as a direct source of glycerol, whereas acetate fulfils the same function indirectly only after its incorporation into carbohydrate. On this ground glucose might be regarded as a better precursor of 'fat' than acetate.

*Mode of conversion of carbohydrate into fat.  
Breakdown of glucose to acetate*

The fact that labelled glucose gave rise not only to labelled fatty acids but also to labelled cholesterol, suggested that glucose is broken down in the body to acetate. This was the more likely since it was shown by Bloch & Rittenberg (1944) that only those substances which gave rise to  $C_2$  compounds were used for the biosynthesis of cholesterol. The only exception to this rule appears to be *isovaleric acid* (Zabin & Bloch, 1950).

We tested this idea by experiments in which [ $^{14}C$ ]acetate or [ $^{14}C$ ]glucose, together with *p*-aminobenzoic acid, were administered to normal and lactating rabbits. It is known that foreign amines, like *p*-aminobenzoic acid, sulphonamides and others, are excreted in the urine as the acetylated compounds (Harrow & Mazur, 1932) and that acetate is an effective acetylating agent for these substances (Bernhard, 1940). In particular, it is thought that *p*-aminobenzoic acid is acetylated only by acetate (Anker, 1948*b*). If, therefore, glucose is broken down in the body to acetate, then after the administration of [ $^{14}C$ ]glucose together with *p*-amino-

Table 1. *Specific activity of glyceride fatty acids and of cholesterol in mammary gland and liver of lactating rabbits 6 hr. after administration of [ $Me-^{14}C$ ]acetate and of [ $^{14}C$ ]glucose*

Exp. no.		Volatile fatty acids		Non-volatile fatty acids		Cholesterol ( $10^{-5} \mu\text{C}/\text{mg. C}$ )	Substance and dose administered
		Soluble in water ( $10^{-3} \mu\text{C}/\text{mg. C}$ )	Insoluble in water	Saturated ( $10^{-5} \mu\text{C}/\text{mg. C}$ )	Unsaturated		
29	Mammary gland	2.30	2.35	23.00	45.00	26.00	{ [ $Me-^{14}C$ ]Acetate 8.3 mg.; 100 $\mu\text{C}$ .
	Liver	—	—	0.90	1.00	15.00	
31	Mammary gland	1.65	1.98	2.40	7.50	5.00	{ [ $^{14}C$ ]Glucose 653 mg.; 91 $\mu\text{C}$ .
	Liver	—	—	0.10	0.16	0.70	

Table 2. *Specific activities of milk constituents (counts/min./C in infinite thickness) from rabbits after administration of [ $\text{carboxy-}^{14}C$ ]acetate or [ $^{14}C$ ]carbohydrate*

Exp. no.	Labelled substance and dose administered	Lactose	Glycerol	Steam-volatile fatty acids		Non-volatile fatty acids	Cholesterol
				Soluble in water	Insoluble in water		
17	Acetate (4.1 mg.; 50 $\mu\text{C}$ )	539	486	2880	2760	355	234
	Starch (37.3 mg.; 50 $\mu\text{C}$ )	11 760	13 332	2855	2740	276	372
18	Acetate (4.1 mg.; 50 $\mu\text{C}$ )	161	—	2068	2158	432	169
	Glucose* (357 mg.; 50 $\mu\text{C}$ )	5 560	3 883	1430	1520	324	299

\* Milk collected during 12 hr., all others 6 hr.

Table 3. *Specific activity of the carbon of 'acetyl', liver fatty acids and liver cholesterol after the administration of [carboxy-<sup>14</sup>C]acetate and of [<sup>14</sup>C]glucose to normal rabbits for 3 days*

(The total dose of each substance was divided into six equal parts which were given to the animals by stomach tube twice daily; the total <sup>14</sup>C dose administered was 75  $\mu$ c. with each substance.)

Substances administered	Specific activity ( $10^{-8}$ $\mu$ c. <sup>14</sup> C/mg. C)			$\frac{(II)}{(I)} \times 100$	$\frac{(III)}{(I)} \times 100$
	'Acetyl' (I)	Fatty acids (II)	Cholesterol (III)		
CH <sub>3</sub> <sup>14</sup> CO <sub>2</sub> Na	7.96	0.144	0.506	1.81	6.36
[ <sup>14</sup> C]Glucose	2.84	0.054	0.167	1.90	5.88

Table 4. *Specific activity of the carbon of 'acetyl' excreted with p-aminobenzoic acid, milk lactose and of glycerol prepared from glycerides of milk and liver of two lactating rabbits 6 hr. after administration of [<sup>14</sup>C]glucose*

(In Exp. no. 31, 653 mg. of glucose uniformly labelled and containing a total of 91  $\mu$ c. of <sup>14</sup>C were injected intravenously as a 5% solution. Immediately before the injection 1 g. of p-aminobenzoic acid (as the sodium salt) was given by stomach tube.

In Exp. no. 36, 10 mg. of labelled glucose, containing 30  $\mu$ c. of <sup>14</sup>C and 1 g. of p-aminobenzoic acid (as the sodium salt) were given together by stomach tube.)

Exp. no.	Specific activity ( $10^{-8}$ $\mu$ c. <sup>14</sup> C/mg. C)				
	Acetyl (I)	Lactose (II)	Glycerol		$\frac{(II)}{(I)} \times 100$
			From milk fat	From liver fat	
31	1.273	6.25	4.78	4.57	20.4
36	0.447	2.12	1.00	0.63	21.1

benzoic acid the animal should excrete [*Ac*-<sup>14</sup>C]*p*-acetamidobenzoic acid.

Table 3 shows that the normal rabbit excretes radioactive acetyl groups after the administration of [<sup>14</sup>C]glucose just as after [<sup>14</sup>C]acetate. Moreover, the ratio of the specific activity of the fatty acids and of cholesterol in the liver to the specific activity of the acetyl groups excreted after the administration of [<sup>14</sup>C]glucose was almost identical with that found after the administration of [<sup>14</sup>C]acetate (see last two columns of Table 3). It is concluded, therefore, that in the rabbit glucose is converted to fatty acids and to cholesterol after being broken down to acetate.

#### *The fraction of body acetate derived from glucose*

It was of interest to determine the proportion of body acetate which is derived from glucose. This can be calculated if it is possible to compare, after the administration of [<sup>14</sup>C]glucose and of *p*-aminobenzoic acid, the <sup>14</sup>C content of the excreted acetyl group with that of the glucose metabolized during the experiment (i.e. in this case with the <sup>14</sup>C content of the precursor of the acetyl group). Ideally, the specific activity of blood glucose should probably be used for such purpose, but for the preparation of glucose (as the phenyllosazone) from blood at least 10 ml. samples are needed; however, the drawing of several samples of this size within a short time from a rabbit would very likely prejudice the experi-

mental results. The lactating animal was thought, therefore, to be a good experimental subject for our purpose, because it secretes into the milk lactose which is derived mostly from blood glucose. It has been shown that after the administration of [<sup>14</sup>C]glucose to lactating rabbits, the specific activities of the glucose and galactose moieties of lactose were equal (French, Popják & Malpress, 1952). It seemed to us a reasonable assumption therefore that the specific activity of lactose would represent the average specific activity of blood glucose and hence of the glucose metabolized during the experimental period. In a few experiments glucosazone was prepared from the blood of animals which were given [<sup>14</sup>C]glucose, [<sup>14</sup>C]acetate, or [<sup>14</sup>C]pyruvate. The specific activity of blood glucose was within 10% identical with that of lactose thus substantiating the above assumption.

Table 4 shows that 6 hr. after the administration of [<sup>14</sup>C]glucose the specific activity of the acetyl C excreted during the experimental period in the urine with *p*-aminobenzoic acid was about 20% of the specific activity of lactose. Since lactose is secreted also continuously, its specific activity is an average for the experimental period just as is that of the acetyl C. It may be concluded, therefore, that about 20% of that part of the body's acetate, which was used for acetylation, was derived from carbohydrate in these animals. It should be emphasized that our animals were not fasted before the experi-

Table 5. *Specific activities (counts/min./C in infinite thickness) of fatty acids of milk glycerides and of cholesterol in rabbits after administration of substances labelled with  $^{14}\text{C}$* (The total radioactive dose with each substance was 50  $\mu\text{c}$ .)

Substance administered	Volatile fatty acids		Non-volatile fatty acids	Cholesterol
	Soluble in water	Insoluble in water		
$\text{CH}_3\text{-}^{14}\text{COOH}$	2068	2158	432	169
$^{14}\text{CH}_2\text{-COOH}$	2605	2825	413	299
$\text{CH}_3\text{-}^{14}\text{CO}\cdot\text{COOH}$	2144	2601	283	175
$^{14}\text{CH}_2\text{-CO}\cdot\text{COOH}$	2000	2160	300	128
$[^{14}\text{C}]\text{Starch}$	2125	2000	110	166
$[^{14}\text{C}]\text{Glucose}$	1430	1520	324	299
$[^{14}\text{C}]\text{Glucose}$	2260	2380	67	—

ment, and consequently the specific activity of liver glycogen could not be used as a measure of the glucose metabolized. We have experimental evidence to show that in a rabbit fed *ad lib.* it may require several days before the liver glycogen becomes equilibrated with blood glucose, and furthermore that the animal metabolizes first carbohydrate absorbed from the diet. For example, the specific activity of the liver glycogen in five experiments out of six was very much lower than that of milk lactose or that of the glycerol of liver triglycerides, which shows that glucose with a higher specific activity than that of liver glycogen was metabolized.

Pyruvate has been thought for a long time to be an intermediate in the conversion of carbohydrate to fatty acids, and since the most probable pathway of acetate formation from glucose seemed to be the oxidative decarboxylation of pyruvate, a further comparison was made between acetate, pyruvate and glucose as precursors of milk fatty acids. The results of such a comparison are shown in Table 5, from which it is evident that the amounts of  $^{14}\text{C}$  incorporated from all three labelled substances into fatty acids and into cholesterol were essentially the same. Considering the fact that each experiment recorded in Table 5 was carried out on a different animal the radioactive counts show remarkably small variation. This might be purely fortuitous or it might mean that the metabolic pool of fatty-acid precursor became labelled to the same extent from the administered  $[^{14}\text{C}]\text{pyruvate}$  and  $[^{14}\text{C}]\text{glucose}$  as from  $[^{14}\text{C}]\text{acetate}$ . It was therefore necessary to compare the specific activity of the milk fatty acids with the specific activity of the excreted acetyl group after the administration of labelled acetate, pyruvate and glucose. While it is very uncertain to what extent the radioactivity of 'acetyl' C represents the radioactivity of acetate in the mammary gland, nevertheless the comparison may act as a useful guide to the approximate amount of the fatty acids synthesized in the mammary gland from acetate. The values shown in Table 6 indicate that in 6 hr. 30–70% of the short-chain fatty acids (up to

about  $\text{C}_{12}$ ) might be derived from acetate provided that the specific activity of the excreted 'acetyl' C represents the specific activity of acetate in the mammary gland. Whether or not this assumption is correct cannot be determined at present. It is to be

Table 6. *Specific activity of short-chain fatty acids in milk fat as a percentage of the specific activity of acetyl group excreted with p-aminobenzoic acid 6 hr. after administration of acetate, pyruvate and glucose labelled with  $^{14}\text{C}$* 

Substance administered	Short-chain fatty acids	
	Soluble in water	Insoluble in water
$[\text{carboxy-}^{14}\text{C}]\text{Acetate}$	51.2	55.6
$[\text{Me-}^{14}\text{C}]\text{Acetate}$	29.6	32.0
$[^{14}\text{C}]\text{Glucose}$	64.4	68.9
$[1\text{-}^{14}\text{C}]\text{Glucose}$	42.0	52.5
$[\alpha\text{-}^{14}\text{C}]\text{Pyruvate}$	34.0	38.5
$[\beta\text{-}^{14}\text{C}]\text{Pyruvate}$	63.7	68.9

Table 7. *Specific activity of short-chain fatty acids in milk fat as a percentage of the specific activity of lactose 6 hr. after administration of  $[^{14}\text{C}]\text{glucose}$  to lactating rabbits*

Exp. no.	Short-chain fatty acids	
	Soluble in water	Insoluble in water
16	26.3	21.8
17	24.2	23.3
18	25.7	27.4
31	26.3	31.6

noted that the figures in Table 6 show a much greater variation than the figures in Table 5. This fact suggests to us that the radioactivity of the 'acetyl' C excreted in the urine does not really represent the radioactivity of the metabolically active acetate in the mammary gland. In the experiments in which  $[^{14}\text{C}]\text{glucose}$  was administered the radioactivity of the short-chain fatty acids was about 25% of that of milk lactose (Table 7). This value can be interpreted with much greater confidence to mean that in 6 hr. 25% of the short-chain fatty acids were derived from carbohydrate.

Table 8. *Distribution of  $^{14}\text{C}$  in milk octanoic acid and in 'acetyl' excreted in the urine after administration of substances labelled with  $^{14}\text{C}$* 

Substance administered	Acetyl carbon atom		Octanoic acid					
	1	2	Carbon atoms 3-8 together (Determined value)	Carbon atoms 3-6 together (Calculated value)	Carbon atom no.			
					1	2	7	8
[ <i>carboxy-<math>^{14}\text{C}</math></i> ]Acetate	>99.0	<1.0	50	50	>99	<1	>99	<1
[ $\alpha$ - $^{14}\text{C}$ ]Pyruvate	96.5	3.5	50	50	96	4	96	4
[ <i>Me-<math>^{14}\text{C}</math></i> ]Acetate	2.0	98.0	50	50	2	98	2	98
[ $\beta$ - $^{14}\text{C}$ ]Pyruvate	3.7	96.3	50	50	4	96	4	96
[1- $^{14}\text{C}$ ]Glucose	3.6	96.4	50	50	5.8	94.2	5.8	94.2

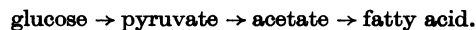
*Distribution of  $^{14}\text{C}$  in fatty-acid chain after administration of specifically labelled precursors*

Whether or not carbohydrate is converted into fatty acids through a  $\text{C}_2$  compound derived from the decarboxylation of pyruvate, can be tested with specifically labelled substances. According to the known reactions of the glycolytic cycle glucose is broken down to pyruvate in such a way that carbon atoms 1 and 6 of glucose will become the  $\beta$ -carbon of pyruvate, and carbon atoms 3 and 4 will become the carboxyl carbon of pyruvate. Pyruvate resulting from the metabolism of [1- $^{14}\text{C}$ ]glucose should be labelled, therefore, in the  $\beta$ -carbon atom; on decarboxylation this pyruvate should yield acetate labelled in the  $\alpha$ -carbon. Whether or not these reactions occur in the animal can be determined by comparing the position of the carbon label in a fatty acid or in the acetyl group excreted with a foreign amine after the administration of appropriately labelled acetate, pyruvate and glucose. The distribution of the label in a fatty acid is being used in this case as an indicator of metabolic reactions in the same way as the labelling of glucose has been employed in the study of intermediary metabolism (cf. Wood, 1948).

Octanoic acid was chosen as the 'indicator' because it is relatively abundant in the milk and mammary-gland fat of rabbits, and also because it is among the acids of the highest specific activity under our experimental conditions. The biological samples could be diluted thus several fold by inactive octanoic acid when necessary so that the chemical degradation could be carried out on samples of convenient size.

The results of degradations of the acetyl group excreted in the urine with *p*-aminobenzoic acid and of the partial degradations of octanoic acid of milk and mammary-gland fat after the administration of  $\text{CH}_3$ . $^{14}\text{COOH}$ ,  $^{14}\text{CH}_2$ . $\text{COOH}$ ,  $\text{CH}_2$ . $^{14}\text{CO}$ . $\text{COOH}$ ,  $^{14}\text{CH}_2$ . $\text{CO}$ . $\text{COOH}$ , and of [1- $^{14}\text{C}$ ]glucose are shown in Table 8. The specific activities of the carbon atoms in this table are expressed in arbitrary units, the average specific activity of all the carbon atoms of the molecule together being taken as 50. The

absolute values of the specific activities of the carbon atoms are, of course, irrelevant from the point of view of the present discussion. It can be seen from Table 8 that [*carboxy- $^{14}\text{C}$* ]acetate and [ $\alpha$ - $^{14}\text{C}$ ]pyruvate gave rise to fatty acids labelled in the odd-numbered carbon atoms, and [*Me- $^{14}\text{C}$* ] acetate, [ $\beta$ - $^{14}\text{C}$ ]pyruvate and [1- $^{14}\text{C}$ ]glucose yielded fatty acids labelled mainly in the even carbon atoms. It may be concluded therefore that carbohydrate is converted to fatty acids by the overall reactions:



*The derivation of glyceride glycerol from carbohydrate*

It was observed, in our experiments, that after the administration of [ $^{14}\text{C}$ ]glucose (or starch) to a lactating animal, blood glucose and milk lactose had the highest specific activity among the various body constituents studied and that the specific activity of

Table 9. *Specific activity (counts/min./C in infinite thickness) of lactose and of neutral-fat glycerol in milk of lactating rabbits 6 hr. after administration of various substances labelled with  $^{14}\text{C}$* 

Substance and dose administered	Specific activity of (II) $\times 100$		
	Lactose (I)	Glycerol (II)	(I)
[ $^{14}\text{C}$ ]Starch; 12 $\mu\text{c}$ .	1 385	965	70.0
[ $^{14}\text{C}$ ]Starch; 50 $\mu\text{c}$ .	11 760	13 332	113.4
[ $^{14}\text{C}$ ]Starch; 50 $\mu\text{c}$ .	6 960	4 180	80.0
[ $^{14}\text{C}$ ]Glucose; 50 $\mu\text{c}$ .	5 560	3 883	70.0
[ $^{14}\text{C}$ ]Glucose; 100 $\mu\text{c}$ .	15 420	11 800	76.5
[ <i>carboxy-<math>^{14}\text{C}</math></i> ]Acetate; 50 $\mu\text{c}$ .	539	486	90.0
[ <i>Me-<math>^{14}\text{C}</math></i> ]Acetate; 50 $\mu\text{c}$ .	581	376	64.7
[ $\alpha$ - $^{14}\text{C}$ ]Pyruvate; 50 $\mu\text{c}$ .	510	476	93.5
[ $\beta$ - $^{14}\text{C}$ ]Pyruvate; 100 $\mu\text{c}$ .	1 063	971	91.4

the glycerol of milk fat varied between 70 and 100 % of that of lactose. Furthermore, even after the administration of labelled acetate or pyruvate—both of which are known to yield labelled glucose—the specific activity of glycerol varied again between 65 and 90 % of that of lactose (Table 9). These observations can be explained only by assuming the rapid and direct formation of glycerol from carbohydrate in the animal (cf. Popják *et al.* 1952).

## DISCUSSION

*The biosynthesis of fat in the mammary gland*

The experiments have proved that synthesis from small molecules within the mammary gland is of major importance in the origin of milk fat in the rabbit just as it was found to be in the lactating goat (Popják *et al.* 1951). The results showed a further similarity to those obtained on the ruminant in that the short-chain fatty acids had the highest specific activity after the administration of labelled acetate, pyruvate or glucose. This finding confirms the previous conclusion that the short-chain fatty acids in milk originate by a synthetic process and not by the degradation of long-chain acids.

Folley & French (1950) concluded, on the basis of respiratory measurements of tissue slices *in vitro*, that mammary-gland slices of rabbits utilized glucose but not acetate for fat synthesis. The equal ease with which fatty acids are synthesized both from acetate and from glucose in the mamma of the intact rabbit might be, therefore, surprising. However, Folley & French (1950) found that, in the presence of glucose, acetate was also used for fatty-acid synthesis. Of course, in the intact animal both substrates are readily available. Since glucose is converted into fatty acids after its breakdown to acetate, it seems probable that the differences observed in respect of utilization of acetate and glucose by tissue slices *in vitro*, when these two substrates are added separately to the incubation medium, are related to the energy-yielding mechanism required for the activation of acetate (cf. Popják, 1952*a*; Folley, 1952).

In the experiment on a lactating goat, already referred to, the data were insufficient for the calculation of the amounts of fatty acids synthesized from acetate in the udder. The only quantitative data obtained showed that in 6 hr. 50% of the injected acetate not oxidized was converted into milk fatty acids. In view of the controversy centring around the origin of short-chain fatty acids in milk fat (cf. Folley, 1949; Achaya & Hilditch, 1950; Hilditch, 1952), the quantitative data obtained in this investigation are of interest, although they are by no means clear-cut. There are three values to be reconciled with one another. The comparison of the specific activity of the acetyl carbon, excreted in the urine, with that of the short-chain fatty acids in the mammary-gland fat indicated that 30–70% of these acids might have been synthesized from acetate (1). The comparison of the radioactivity of milk lactose with that of the short-chain fatty acids after the administration of labelled glucose showed that about 25% of these fatty acids were formed from glucose (2). On the other hand, 20–21% of the body's acetate used for acetylation

was also derived from glucose (3). Clearly, the quantities mentioned under (1), (2) and (3) are somewhat conflicting. However, conclusion (1) is based on the assumption that the radioactivity of the acetyl C excreted in the urine represents also the radioactivity of the acetate metabolized in the mammary gland. This is by no means certain, and in fact seems to us rather doubtful. If this assumption were correct, then, since about 20% of the acetyl group is derived from glucose, the amount of the short-chain fatty acids synthesized from carbohydrate should have been found to be between 6 and 14%. The higher value found (25%) and the conclusion that glucose is converted to fatty acids by way of acetate (see later), suggests very strongly that the specific activity of the acetate in the mammary gland was significantly lower than that of the acetate used in the acetylation of *p*-aminobenzoic acid. It seems therefore that we are underestimating the percentage of short-chain fatty acids synthesized from acetate.

*Conversion of carbohydrate to fat*

*Conversion into fatty acids.* The experimental results presented have shown that glucose is broken down to acetate and that in the liver the ratio of the specific activities of glyceride fatty acids and of cholesterol to the specific activity of the acetyl group excreted in the urine was very similar whether labelled glucose or labelled acetate were administered to the animals. Since the most probable site of acetylation of *p*-aminobenzoic acid is the liver, it is a reasonable assumption that the radioactivity of the acetyl group represents the radioactivity of the metabolically active acetate in the liver from which fatty acids and cholesterol are synthesized. It is concluded, therefore, that glucose is converted to fatty acids and to cholesterol via acetate.

The results obtained by the degradation of fatty acids and of the excreted acetyl support in every way this conclusion. It was found that [*Me*-<sup>14</sup>C]-acetate, [ $\beta$ -<sup>14</sup>C]pyruvate and [1-<sup>14</sup>C]glucose all yielded fatty acids labelled in a similar way, about 95% of the isotope content being confined to the even-numbered carbon atoms of the fatty acid chain; [*carboxy*-<sup>14</sup>C]acetate and [ $\alpha$ -<sup>14</sup>C]pyruvate, on the other hand, gave fatty acids labelled in the odd-numbered carbon atoms. Fatty acids formed from [2-<sup>14</sup>C]glucose, which we have not been able to test as yet, should be labelled as the acids after the administration of [*carboxy*-<sup>14</sup>C]acetate or of [ $\alpha$ -<sup>14</sup>C]pyruvate. Our conclusion is, therefore, that glucose is broken down in the glycolytic cycle to pyruvate; the oxidative decarboxylation of the latter yields acetate, which forms then the building unit for the synthesis of fatty acid and of cholesterol. The various hypotheses of possible



mechanisms by which fatty acids with a chain of an even number of carbon atoms could be formed from pyruvate have been reviewed by Breusch (1952). Rittenberg & Bloch (1945) thought that the formation of acylpyruvic acids followed by decarboxylation might provide a more direct conversion of carbohydrate to fatty acids than the mechanism we suggest here. Anker (1948*b*) supported Rittenberg & Bloch (1945) in their view on the basis of finding in one strain of rats only very small amounts of isotope in *p*-acetamidobenzoic acid after the administration of pyruvate labelled in either the  $\alpha$ - or  $\beta$ -carbon atom, although the acetyl group excreted with sulphanilamide and the fatty acids were highly labelled. In another strain of rats, however, radioactive acetyl group was excreted both with *p*-aminobenzoic acid and with sulphanilamide after the administration of  $\alpha$ - or  $\beta$ -labelled pyruvate. These results of Anker (1948*b*) could be explained equally well on the assumption that in the two strains of rats the acetylation of the two amines took place at different sites rather than being indicative of a direct utilization of pyruvate for fatty-acid synthesis. In our animals the conversion of both pyruvate and of glucose to acetate was so great as judged by the acetylation of *p*-aminobenzoic acid that we have no reason to suspect that, in the rabbit, glucose or pyruvate act as precursors of fatty acids otherwise than by virtue of their previous breakdown to acetate.

The figures in Table 8 show that all the  $^{14}\text{C}$  in the fatty acids (or in the acetyl group) was not confined entirely to the alternate carbon atoms after the administration of [*Me*- $^{14}\text{C}$ ]acetate, [ $\beta$ - $^{14}\text{C}$ ]pyruvate or of [ $1$ - $^{14}\text{C}$ ]glucose. The metabolism of pyruvate and acetate via the citric acid cycle provides a means for the rearrangement of the label in these substances. That this is not purely a speculative view was shown, for example, by the degradation of the glucose unit of lactose isolated from the milk of a rabbit after the administration of [ $1$ - $^{14}\text{C}$ ]glucose. The degradation, carried out by Dr H. G. Wood and to be published later, showed that significant amounts of radioactivity were present in carbon atoms 3, 4 and 2, 5 in addition to 1, 6.

*The formation of glycerol from glucose.* Our results give some new information on a hitherto neglected aspect of the biosynthesis of fat in animals, i.e. on the origin and turnover of glycerol. The results presented in this and in a previous paper (Popják *et al.* 1952) demonstrate for the first time the formation of glycerol from glucose in the animal organism. The specific activity of glycerol in the present experiments varied between 65 and 95% of that of milk lactose. Since it was found previously that the specific activity of the glucose and galactose units in lactose are equal after the administration of [ $^{14}\text{C}$ ]glucose (French *et al.* 1952),

we interpret our findings to mean that 65–95% of the milk and mammary-gland fat was renewed within the experimental period. On the basis of the arguments presented earlier it seems probable that about the same percentage of the short-chain fatty acids was also newly synthesized in the mammary gland. However, the glycerol was derived from the mixed glycerides containing all types of the fatty acids and it should be recalled that the specific activity of the long-chain acids was only about one-fifth to one-tenth of that of the short-chain fatty acids (cf. Tables 2 and 5). The nearly complete renewal of glycerol in the mammary gland within 6 hr. is therefore somewhat surprising. The only interpretation that can be given at present to this finding is that the rate of renewal of glycerol and of fatty acids in glycerides is not equal and that the glycerol in the mammary gland is being metabolized in addition to providing a structural part of fat. In a previous experiment on a lactating goat (Popják *et al.* 1951) the half-life of milk-glyceride fatty acids was calculated to be 4 hr. (cf. Popják, 1952*a*) and that of the glycerol 3 hr. (Popják *et al.* 1952). The disparity between the rate of renewal of glycerol and of fatty acids was apparent in our experiments to an even greater extent in liver fat. It appears therefore that the glycerol part of fat is metabolized by the body faster than the fatty acid part.

Taking all our results together it is evident that the conversion of carbohydrate into fat means not only the formation of fatty acids but of the entire molecule, including glycerol.

## SUMMARY

1. The biosynthesis of fat was studied in lactating rabbits with the aid of acetate, pyruvate and glucose labelled with  $^{14}\text{C}$ .
2. It has been shown that both short- and long-chain fatty acids and cholesterol are synthesized from acetate and from carbohydrate in the mamma of lactating rabbits.
3. It was estimated that in 6 hr. at least 30–70% of the short-chain acids originate from acetate, or about 25% from carbohydrate; only one-fifth to one-tenth of these amounts of the long-chain acids were, however, synthesized in the mammary gland from either of these sources.
4. Some 65–95% of the glycerol part of fat, derived from carbohydrate, was newly formed in the mammary gland in 6 hr. It is concluded that the glycerol part of fat is more rapidly metabolized by the animal than the fatty-acid part.
5. About 20% of the body's acetate used for acetylation of *p*-aminobenzoic acid was derived from glucose in fully fed lactating rabbits.
6. Octanoic acid from milk and mammary-gland fat and the acetyl group of *p*-acetamidobenzoic acid

were degraded chemically after the administration of [*carboxy*-<sup>14</sup>C] and [*Me*-<sup>14</sup>C]acetate, [ $\alpha$ -<sup>14</sup>C] and [ $\beta$ -<sup>14</sup>C]pyruvate and of [*1*-<sup>14</sup>C]glucose.

7. The results of degradations showed that the conversion of glucose to fatty acids proceeds by the overall reactions equivalent to glucose  $\rightarrow$  pyruvate  $\rightarrow$  acetate  $\rightarrow$  fatty acid.

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## Urinary Porphyrins in Lead-treated Rabbits

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New chromatographic methods of identifying and separating porphyrins (Nicholas & Rimington, 1949, 1951; Nicholas & Comfort, 1949; Nicholas, 1951; Chu, Green & Chu, 1951) have made possible the detection of previously undescribed porphyrins in biological material. The ether-soluble porphyrins found in the urine of men and of animals exposed to lead have been regarded until recently as consisting mainly, if not entirely, of coproporphyrin III. However, a tricarboxylic porphyrin has recently been found (Nicholas & Rimington, 1951) in the urine of a case of industrial lead poisoning and in a preparation of supposed coproporphyrin from the

urine of lead-treated rabbits, as well as from other sources. Di-, penta-, hexa- and hepta-carboxylic porphyrins were found by the same workers in other pathological conditions. Weatherall & Comfort (1952) have also described tri- and tetra-carboxylic porphyrins, and have obtained evidence for the existence of a pentacarboxylic porphyrin in the urine of lead-poisoned rabbits, and have found similar porphyrins in the urine of normal rabbits. Kench, Lane & Varley (1952*a, b*) have found coproporphyrin I as well as coproporphyrin III, and an uncharacterized porphyrin in the urine of men suffering from lead poisoning. We have examined the urinary porphyrins of lead-treated rabbits in more detail and the results are described in this paper.

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