Synthesis of Cholesterol and Fatty Acids in Foetuses and in Mammary Glands of Pregnant Rabbits

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The aim of this investigation was to study the sources of foetal fatty acids and cholesterol, i.e. to decide whether these substances are derived from the mother by placental transmission or are synthesized within the foetus from suitable precursors. Further, it was hoped that by studying foetal organs separately information might be obtained about their role in the fat metabolism of the foetus.

It was shown in previous investigations (Popják, 1947; Popják & Beeckmans, 1950) that phospholipins in the rabbit foetus are formed by foetal synthesis and that although the placenta of foetal origin absorbs much phospholipin from the maternal blood it does not transmit unhydrolysed phospholipin molecules to the body of the foetus. Certain observations on the fat content of foetuses from cholesterol-fed mothers could be explained on the assumption that fatty acids and cholesterol are also chiefly synthesized within the foetus from smaller molecules, and it was pointed out that this problem could be solved with the aid of isotopes (Popják, 1946). While this investigation was in progress Goldwater & Stetten (1947) provided evidence that fatty acids and cholesterol are mainly synthesized in the rat foetus, as judged by the rapid incorporation of deuterium (D) into these substances from the body water. In addition they showed that, when D-labelled fatty acids were fed to the mothers for 2 days (between the eighteenth and the twentieth days of gestation), not more than 1·5 % of the foetal fatty acids were derived from the maternal dietary fat. When deuteriocholesterol was fed for 7 days (between the thirteenth and twentieth days of gestation) to pregnant rats, approximately 10·7 % of the foetal cholesterol was derived from the dietary cholesterol.

The results of this investigation, carried out on rabbits, generally confirm the findings of Goldwater & Stetten (1947) on rats and provide additional proof by showing the utilization of acetate-carbon for fatty acid and cholesterol synthesis in the foetus. The said authors, however, studied only the unfractionated total fatty acids and cholesterol in the foetus as a whole and their investigation gives no information about the role of the placenta, liver or extrahepatic tissues of the foetus in lipid synthesis. The question whether foetal lipids are synthesized within the body of the foetus or in the placenta and transported from there was left entirely open. In the present investigation particular attention was paid to these points. Further, in a quantitative analysis of the results a comparison was made between the rate of accumulation and rate of synthesis of the fatty substances in the growing foetus in order to assess whether growth is associated primarily with increased synthetic activity or decreased degradation, or both.

The classical experiments of the Schoenheimer school (Schoenheimer, 1941) have shown that the rate of incorporation of D into fatty acids and cholesterol in animals whose body water has been enriched with D_2O for varying lengths of time, gives a good measure of the rates of synthesis and degradation of these compounds. The first part of the present investigation is concerned with the rate of synthesis of D into foetal cholesterol and fatty acids. In later sections the utilization of acetate in the foetus for the synthesis of these compounds will be demonstrated, and observations on fat formation in the mammary gland of pregnant, non-lactating rabbits will also be reported.

To obtain evidence concerning the question whether the foetal lipids are derived from the preformed maternal compounds by placental transmission or by synthesis within the foetus, a comparison between the isotope content of the maternal plasma lipids and foetal lipids should be made. If the latter were to exceed the former under the experimental conditions to be outlined, the result would indicate foetal synthesis. Unfortunately, material sufficient for D analysis by the falling-drop method used in this investigation, cannot be obtained from the plasma of one rabbit, particularly as rabbits develop a lipopenia during the second half of pregnancy (Baumann & Holly, 1925–6; Popják, 1946).

The D content of fatty acids and cholesterol of the maternal liver was therefore compared with that of the foetal tissues on the assumption that the plasma lipids are derived from the liver or are in equilibrium with liver lipids. If this assumption is correct, then under our experimental conditions the D content of the maternal plasma lipids cannot exceed that of the liver lipids. It is known that plasma phospholipins in the adult animal are synthesized in the liver (Fishler, Entenman, Montgomery & Chaikoff, 1943;

Entenman, Chaikoff & Zilversmit, 1946). Results obtained in animals injected with $CH_3{}^{14}COONa$ indicate that the source of plasma cholesterol is also probably the liver. The available evidence, therefore, seems to justify the assumption on which the interpretations of the results of this investigation are based.

METHODS

Rabbits (2·5–3·0 kg.) were inseminated artificially, and hence the period of gestation was known within narrow limits. They were first injected with gonadotrophic hormone (50 i.u.) and were inseminated (0·2 ml. of semen) within 1 hr. Under such conditions conception should take place during the 12 hr. period after the injection of gonadotrophic hormone.

On a chosen day during pregnancy (between the 16th and 28th day) the animals were given intravenously an amount of pure D_2O (made isotonic with NaCl) which brought the concentration of D_2O in the body fluids to 1·5%; the drinking water was then replaced by 2% D_2O in tap water. The concentration of D_2O in the body fluids was thus maintained constant at a level of about 1·5% for 1–12 days. All experiments were terminated on the 28th day of pregnancy. If, therefore, the animals were kept on D_2O for n days, the beginning of the deuterium regimen was on the $(28-n)$th day. This particular mode of administration of D_2O to a growing system, like the foetus, allows a comparison between the rate of synthesis and the rate of accumulation of a particular substance, as will be shown in the discussion. The alternative choice of giving D_2O for n days starting, say, on the 16th day and ending the experiment on the $(16+n)$th day is unsuitable for such purpose.

On the 28th day of pregnancy the animals were anaesthetized with nembutal and ether and the foetuses delivered one by one as described previously (Popják, 1946). Foetal blood was obtained from the umbilical vein and maternal from the aorta; both were heparinized.

Preparation of tissues. The excised livers of foetuses from each litter were pooled; carcasses were also pooled. The placentae were freed from the membranes and the foetal and maternal parts separated. Only the foetal part, i.e. that derived from the ovum, was investigated systematically. The lipids of the maternal portion of the placenta were examined only in two experiments, in which the animals were injected with $CH_3{}^{14}COONa$. The mammary glands of the mothers were dissected out and freed as far as possible of connective and adipose tissue.

All tissues were minced and ground to a fine pulp; the foetal carcasses were first minced and then reduced to a pulp in a Waring blender.

Samples were taken from the maternal liver and blood, the placenta, foetal liver and carcass for the distillation of body water in a high vacuum system. The D content of these water samples was determined and found to be identical within experimental errors.

Extraction and fractionation of lipids. The pulped tissues were extracted four times by boiling with ethanol-ether (3:1, v/v) for 5 min. each. The total volume of solvent used was at least 30 times the weight of the tissue. Negligible amounts of fatty material remain in the tissues after such extraction. The filtered and combined extracts were evaporated to dryness under reduced pressure at 30–40° in an atmosphere of N_2. The residues were extracted with boiling light petroleum (b.p. 40–60°) and the extract freed from suspended matter by centrifuging. The clear petroleum extracts were concentrated to a suitable small volume and the phospholipins precipitated by the addition of acetone and a saturated ethanolic solution of $MgCl_2$. The phospholipins were allowed to settle overnight in the refrigerator. After centrifuging, the acetone supernatant was poured off. The phospholipins were taken up in light petroleum and re-precipitated.

The phospholipins were dissolved in 10–20 ml. of moist ether and saponified by the addition of an equal volume of 80% ethanol and 40% (w/v) aqueous KOH (5 ml./g. of lipid) at 70–80° for 24 hr. As the saponification progressed, more and more water was added and at the end all the organic solvent was evaporated off. The soaps were transferred with hot water to separatory funnels, made acid with H_2SO_4 and the fatty acids extracted with light petroleum.

The acetone supernatants, obtained from the phospholipin precipitations and containing glycerides, together with free and combined cholesterol, were evaporated to dryness by distillation and the residues saponified with ethanolic KOH at 80° for 6 hr.; 10 ml. of ethanol and 1 ml. of 40% (w/v) aqueous KOH were used/g. of glyceride fat. When the saponification was finished the material was transferred with hot 50% (w/v) ethanol to separatory funnels and the unsaponifiable matter removed by extraction with light petroleum. Five petroleum extractions were carried out, after which the organic solvents were evaporated off from the soaps on a water bath. The free fatty acids were obtained as described for phospholipins. The light petroleum was evaporated off and the fatty acids dried over $CaCl_2$ and paraffin. These fatty acids will be designated as glyceride fatty acids, although a very small portion of them were derived from cholesteryl esters also. The glyceride fatty acids obtained from maternal and foetal liver and foetal carcass were also separated into saturated and unsaturated acids by the lead-salt method (Twitchell, 1921).

The extract of unsaponifiable matter was concentrated nearly to dryness, and 5–10 ml. of acetone were added. Cholesterol was precipitated by the addition of 0·5% (w/v) digitonin solution in 80% ethanol. The digitonide was washed twice with acetone-ether (2:1, v/v) and twice with ether and then dried, first at 70° and then *in vacuo* over P_2O_5.

Determination of deuterium in fatty acids and cholesterol. The organic substances were burnt and the water of combustion collected and purified as described by Keston, Rittenberg & Schoenheimer (1937–8). The deuterium contents in the water of combustion and in the water obtained from the distillation of tissue samples were determined by the falling-drop method (Barbour & Hamilton, 1926). *o*-Fluorotoluene was used as the medium through which the drops were allowed to fall. The temperature of the bath was maintained within $\pm 1{\cdot}5 \times 10^{-3}$°. A brief description of the constant-temperature bath and micropipette used for delivery of drops will be given in Appendix 1.

In the calculation of D content of cholesterol from the deuterium content of cholesterol-digitonide it was assumed that in 1 molecule of cholesterol-digitonide 46 H atoms of the cholesterol were diluted with 92 H atoms derived from digitonin, an assumption verified by experiment.

The expression of 'atom-% excess' for the D content of cholesterol and fatty acids will be used in this paper to denote the D content (atom-% excess) of these expressed as the percentage of the D content (atom-% excess) of the body water.

RESULTS

In all our experiments, whether of 1- or 12-day duration, the foetal cholesterol and fatty acids contained a higher concentration of D than the corresponding substances in the maternal liver (Tables 1–4). The results, therefore, generally indicate that fatty acids and cholesterol are synthesized within the foetus, thus confirming the findings of Goldwater & Stetten (1947). There are, however, significant differences in the isotope contents of these substances in the various foetal tissues. The further analysis of the results will be directed towards assessing the role of the foetal liver, the extrahepatic tissues and of the placenta in foetal-fat metabolism.

Synthesis of deuterium into foetal cholesterol. From Table 1 it can be seen that the most rapid incorporation of D from body water into cholesterol occurs in the foetal liver, the next in order being the foetal carcass. In 11 and 12 days the D concentration in the cholesterol of both foetal liver and foetal carcass seems to have reached a maximum concentration of about 63 atom-% excess. Rittenberg & Schoenheimer (1937) have shown that the maximum concentration of D in cholesterol of adult mice was 50% of that found in the body water when the animals were kept on D_2O for as long as 50–100 days.

Table 1. *Deuterium content of cholesterol obtained from tissues of animals whose body water was enriched with* D_2O *for* 1–12 *days between the* $(28-n)$*th and* 28*th day of pregnancy*

(D content of cholesterol (atom-% excess) expressed as the percentage of the D content (atom-% excess) of body water.)

No. of days on D_2O (n)	Cholesterol from			
	Maternal liver	Foetal placenta	Foetal liver	Foetal carcass
1	10·80	10·20	20·50	15·10
2	21·95	22·00	—	29·30
3	24·40	25·65	43·35	35·61
4	29·40	29·40	51·00	43·30
6	32·70	39·00	53·00	50·40
11	33·80	40·20	63·30	64·40
12	48·40	51·00	62·37	63·00

To test whether cholesterol in the foetus is synthesized throughout the development with a maximum isotope concentration of 63 atom-% excess, another experiment was carried out in which a pregnant rabbit was kept on D_2O for 7 days between the 16th and 23rd days of pregnancy. A 16-day rabbit embryo contains about 0·5 mg. and a 23-day foetus 12·5 mg. of cholesterol, i.e. 96% of the amount present on the 23rd day has accumulated between the 16th and 23rd days of intrauterine life. If the 12 mg. which have been added to the 0·5 mg. present in the 16-day embryo had been synthesized within the foetus with a D content of 63 atom-% excess, then the total foetal cholesterol should have a D content of 60·5 atom-% excess. The values found were 61·5 and 62 atom-% excess in foetal liver and carcass-cholesterol respectively and therefore are close to the prediction.

The D content of the cholesterol extracted from the placenta was only slightly more than that found for maternal liver cholesterol (Table 1). The ability of the foetal placenta to synthesize cholesterol was, however, proved definitely by results obtained after the injection of labelled acetate (Tables 5, 6). The low D content found may mean either a relatively slow turnover rate of cholesterol in the placenta, or, in addition to synthesis, absorption of cholesterol with low isotope content from the maternal blood. The absorption of cholesterol from the maternal blood and its storage by the placenta have previously been demonstrated (Popják, 1946). Since the D content of the cholesterol in the placenta was less than that in either the foetal liver or in the foetal carcass, the sterol in the latter tissues could not have been derived from the placenta, but must have been synthesized within the body of the foetus.

Synthesis of deuterium into glyceride and phospholipin fatty acids. The D content of glyceride and phospholipin fatty acids extracted from the maternal liver and foetal tissues is shown in Tables 2 and 3. The fatty acids of the foetal carcass were much richer in the isotope than those of the maternal liver, which proved the synthesis of fatty acids by the foetal tissues. The D content of the fatty acids extracted from the foetal liver and foetal placenta, however, exceeded the isotope content of the maternal liver fatty acids by only a narrow margin.

The interpretation of the results shown in Tables 2 and 3 is made somewhat difficult by the fact that these fatty acids are not homogeneous for it has been shown by Rittenberg & Schoenheimer (1937) that the saturated fatty acids are regenerated in the body much faster, and acquire a higher D content, than the unsaturated acids. It is known that the proportion of the saturated acids in the foetus is higher than in the adult animal (Chaikoff & Robinson, 1933). The results shown in Tables 2 and 3 might therefore be taken to indicate that the maternal saturated fatty acids, which should have a higher isotope content than the unfractionated acids, have been transferred selectively across the placenta and have accumulated in the foetal organs.

To decide if such an interpretation is valid, the glyceride fatty acids from the maternal and foetal liver and foetal carcass were fractionated into saturated and unsaturated acids. The D contents of the two fractions are shown in Table 4. The saturated fatty acids in the foetal carcass contained a higher concentration of isotope than the saturated acids of the maternal liver. In the experiment

recorded in the last line of Table 4, in which the body water of the animal was enriched with D_2O for 12 days, from the 16th to the 28th days of gestation, the D content of the saturated acids obtained from the foetal carcass was 50 atom-% excess. Rittenberg & Schoenheimer (1937) have shown that, during the total synthesis of saturated fatty acids in animals, 50 % of the stably bound H atoms in these acids are derived from the body water (see also Schoenheimer, 1941).

Table 2. *Deuterium content of glyceride fatty acids obtained from tissues of animals whose body water was enriched with* D_2O *for* 1–12 *days between the* $(28-n)$*th and* 28*th day of pregnancy*

(D content of fatty acids (atom-% excess) expressed as the percentage of the D content (atom-% excess) of body water.)

No. of days on D_2O (n)	Glyceride fatty acids from			
	Maternal liver	Foetal placenta	Foetal liver	Foetal carcass
1	4·72	4·19	4·86	5·53
2	4·88	7·41	6·60	11·12
3	5·86	7·00	8·85	14·47
4	7·80	9·02	8·61	18·26
6	12·31	12·80	14·02	25·14
12	22·70	22·65	22·85	33·20

Table 3. *Deuterium content of phospholipin fatty acids obtained from tissues of animals whose body water was enriched with* D_2O *for* 1–12 *days between the* $(28-n)$*th and* 28*th day of pregnancy*

(D content of fatty acids (atom-% excess) expressed as the percentage of the D content (atom-% excess) of body water.)

No. of days on D_2O (n)	Phospholipin fatty acids from			
	Maternal liver	Foetal placenta	Foetal liver	Foetal carcass
1	4·61	4·16	6·66	7·80
2	5·15	6·79	8·64	12·72
3	6·00	8·06	10·55	17·58
4	7·70	9·02	10·20	18·30
6	13·90	10·35	14·63	26·80
12	21·47	20·40	19·30	34·20

The D content of the saturated fatty acids in the foetal liver in the same experiment was, however, only 37·6 atom-% excess. If these fatty acids had been synthesized entirely in the foetal liver by the same biochemical mechanism as those in the foetal extrahepatic tissues, they might be expected to contain similarly 50 atom-% excess deuterium. One obvious explanation is that the fatty acids synthesized in the foetal liver with a high isotope content were diluted with fatty acids low in D from the maternal side. Another possible explanation will be discussed later.

Essentially similar considerations apply to the foetal unsaturated fatty acids. The unsaturated fatty acids in the foetal carcass had a higher isotope content than the corresponding acids in the maternal liver. Except for one experiment, the deuterium content of the unsaturated fatty acids in the foetal liver was, on the other hand, less than in the maternal liver. These results by themselves would therefore make it appear very doubtful that any of these unsaturated acids were synthesized within the foetal liver. It will be shown, with the aid of $CH_3{}^{14}COONa$, that there was definite synthesis of unsaturated acids in the foetal liver.

Fat synthesis in embryonal adipose tissue. It is generally assumed that in the adult animal the liver is the main site of fatty acid synthesis, the extrahepatic tissues being comparatively inert. Although results bearing on this problem, which will be reported separately, indicate that there are tissues in the adult animal which synthesize fatty acids even more rapidly than the liver, it is agreed that the adipose tissues are mainly stores of fat. In the foetal carcass most of the glyceride fat is present in adipose tissue which develops from the mesenchyme. Synthesis of glyceride fatty acids in the extrahepatic foetal tissues has already been demonstrated (Tables 3, 5), and it is thought that the embryonal fatty tissue is the principal organ of this synthesis. This suggestion is borne out by results shown in Table 5. These were obtained on the 23-day pregnant rabbit which was kept on D_2O for 7 days between the 16th and 23rd days of pregnancy, and which was in

Table 4. *Deuterium content of saturated and unsaturated glyceride fatty acids obtained from tissues of animals whose body water was enriched with* D_2O *for* 1–12 *days between the* $(28-n)$*th and* 28*th day of pregnancy*

(D content of fatty acids (atom-% excess) is expressed as percentage of D content (atom-% excess) of body water. The D content of the unfractionated glyceride fatty acids is shown in Table 2. S = saturated and U = unsaturated fatty acids.)

No. of days on D_2O (n)	Maternal liver		Foetal liver		Foetal carcass	
	S	U	S	U	S	U
1	7·60	4·10	6·36	4·10	8·20	3·59
3	6·08	5·59	12·00	7·50	21·64	7·31
4	—*	—*	12·60	5·60	26·80	7·70
6	17·89	9·36	22·90	8·00	39·20	17·02
12	33·40	19·14	37·60	12·00	50·00	31·70

* Insufficient material for fractionation.

Table 5. D *content (atom-% excess) and* ^{14}C *content of maternal and foetal fatty acids in a 23-day pregnant rabbit which was given* D_2O *for 7 days from the 16th to the 23rd day of pregnancy and was injected intravenously with 100 μc. of* ^{14}C *as* $CH_3{}^{14}COONa$ *22 hr. before end of experiment*

(^{14}C content expressed as 1×10^{-5} μc./mg. substance.)

Source	Deuterium content of		^{14}C content of		
	Glyceride fatty acids	Phospholipin fatty acids	Glyceride fatty acids	Phospholipin fatty acids	Cholesterol
Maternal liver	5·3	9·44	2·53	2·66	8·16
Foetal liver	26·5	—*	18·36	14·64	32·40
Foetal carcass†	—*	36·7	13·15	12·64	20·00
Foetal adipose tissue‡	30·6	—	24·10	13·50	21·10
Foetal placenta	—*	—*	6·71	7·76	14·90

* Insufficient material for D determination.

† Denotes the body of the foetus without liver and adipose tissue which have been dissected out.

‡ This represents the earliest fatty tissue visible to the naked eye in the rabbit foetus and which develops as two distinct lobes over and between the scapulae; these fatty lobes continue forward on two sides of the neck which they surround like a collar.

addition injected with $CH_3{}^{14}COONa$ on the 22nd day. This experiment has already been mentioned in connexion with the foetal synthesis of cholesterol. The adipose tissue from the foetuses of this animal was dissected out and its fatty constituents analysed. The especially high degree of utilization of acetate for the synthesis of glyceride fatty acids in this tissue supports the view that the embryonal fatty tissue is one of the main sites of fat formation in the foetus. Unfortunately, sufficient material for fractionation of fatty acids into saturated and unsaturated fatty acids was not available from this experiment.

28th day of gestation and 16 hr. after the last injection of $CH_3{}^{14}COONa$. The ^{14}C content of the fatty acids and cholesterol was determined on the substances themselves with a thin mica-window Geiger-Müller counter as described in Appendix 2. The results of all seven experiments were qualitatively and quantitatively the same, and therefore the details of only one are shown in Table 6, where the ^{14}C contents of foetal cholesterol and fatty acids may be compared with those obtained from the maternal liver and plasma.

Whatever the mode of administration of the labelled acetate, the radioactivity of the maternal

Table 6. ^{14}C *content of cholesterol and fatty acids in maternal and foetal tissues after intravenous injection of 50 μc. of* ^{14}C *as* $CH_3{}^{14}COONa$ *(4·3 mg.) in three doses during 24 hr.*

(^{14}C content expressed as 10^{-5} μc./mg. of substance. M = unfractionated fatty acids; S = saturated fatty acids; U = unsaturated fatty acids.)

Source	Cholesterol	Glyceride fatty acids			Phospholipin fatty acids
		M	S	U	
Maternal liver	8·0	1·39	1·83	0·36	1·11
Maternal plasma	6·5	—	—	—	—
Foetal placenta	11·1	2·2	—	—	2·33
Foetal plasma	19·0	—	—	—	—
Foetal liver	45·6	3·38	5·5	2·0	3·91
Foetal carcass	13·6	2·73	3·66	0·97	2·81

Utilization of acetate for cholesterol and fatty acid synthesis in the foetus. It has been shown by Bloch & Rittenberg (1942*a*, *b*) that acetate is utilized in animals for cholesterol and fatty acid synthesis.

Seven pregnant rabbits, six of which also received D_2O, were injected intravenously with $CH_3{}^{14}COONa$. The total radioactive dose was either 50 or 100 μc. of ^{14}C contained in 4·3 or 8·6 mg. of anhydrous sodium acetate. Either a single intravenous injection was given (as in the experiment shown in Table 5), or the total dose was divided into two or more evenly spaced injections over a period of 1–4 days. In each case the animals were killed on the

plasma cholesterol was always less than that of foetal cholesterol; hence (under our experimental conditions of repeated injections of the labelled precursor) the latter could not have been derived from the former. A comparison of the radioactivities of the cholesterol obtained from the foetal placenta, foetal plasma and foetal liver shows that the highly active cholesterol in the foetal liver was synthesized there and not transported with the foetal plasma from the placenta. These results then confirm in every respect those obtained with the aid of D and show the utilization of acetate C for cholesterol synthesis in the foetus as well as in the adult.

In two experiments the ^{14}C content of cholesterol and fatty acids extracted from the maternal placenta was also examined; they showed just detectable radioactivity (0·5–1·0 count/min. above background), corresponding to a ^{14}C content of less than 10^{-6} μc./mg. of substance. This finding indicates that, at least towards the end of pregnancy, the maternal placenta is metabolically rather inert. The observations suggest further that the source of plasma cholesterol, both in the adult and foetal animal, is the liver, since the ^{14}C content of the plasma cholesterol was closest to that of liver cholesterol both in the mother and the foetus.

Table 6 shows that the isotope content of the fatty acids in the foetal liver and carcass was about two or three times as high as in the maternal liver. Since the radioactivity of the fatty acids in the placenta was higher than in the maternal liver, but lower than in the foetal liver and carcass, it must be concluded that, just as in the case of cholesterol, the fatty acids in the foetal body were mainly synthesized there and not derived from either the placenta or the mother. The results show further that there was a higher percentage utilization of the carboxyl C of acetate for the synthesis of the saturated glyceride acids than for the synthesis of the unsaturated ones, which is a similar finding to that known to be the case for the adult animal (Rittenberg & Bloch, 1945).

Lipid synthesis in the mammary glands of pregnant rabbits. The material of this investigation gave also an opportunity to study the fatty acid and cholesterol metabolism of the developing but non-lactating mammary gland. We wish to record certain observations which form the basis of further experiments already in progress. Table 7 shows the D content of the fatty acids and cholesterol of the mammary glands of pregnant rabbits whose body water was enriched with D_2O for 1–12 days between the 16th and 28th days of pregnancy. As a comparison the values for the liver lipids are also shown in Table 1. It appears that there is synthesis of fatty acids and cholesterol in the mammary gland also, apart from any possible supplies of fatty materials from the liver via the blood stream. There is, however, an interesting feature, which becomes obvious when the data of Table 7 are plotted graphically (Fig. 1). The increases in D content of both phospholipin-fatty acids and cholesterol of the mammary gland show the usual smooth curves. In the case of the glyceride fatty acids, however, there is a sharp break, which appears between the 22nd and 24th days of pregnancy, i.e. between the experiments in which the animals were kept on D_2O for 6 and 4 days respectively. Since this discontinuity is not present in either the cholesterol or phospholipin-fatty acid curves, it cannot be ascribed to either experimental error or biological variations. A tentative explanation is offered, but requires further experimental work. The mammary glands of rabbits are usually well developed by the middle of the gestation period, at about the 16th day of pregnancy, when an active fat secretion begins. If the fats (chiefly glycerides) which accumulate between the 16th and 28th days of pregnancy have been chiefly synthesized by the gland of an animal given D_2O to drink, it is to be expected that the fatty acids should have a high D content. This appears to be the case for the last two experiments shown in Table 7. From the 24th

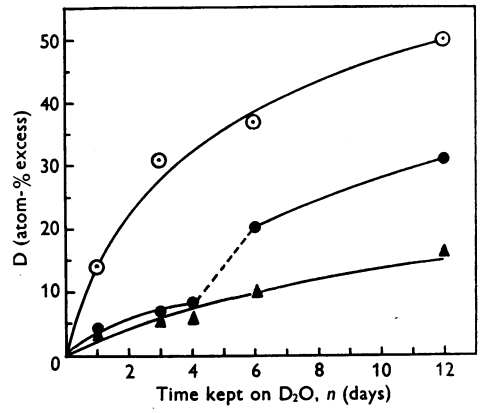

Fig. 1. Incorporation of deuterium into cholesterol and glyceride and phospholipin fatty acids of mammae of non-lactating pregnant rabbits. Cholesterol, ⊙—⊙; glyceride fatty acids, ●—●; phospholipin fatty acids, ▲—▲.

Table 7. *Deuterium content of glyceride and phospholipin fatty acids and of cholesterol in mammary glands and liver of pregnant rabbits whose body water was enriched with* D_2O *for* 1–12 *days between the* $(28-n)$*th and* 28*th day of pregnancy*

(D content of lipids (atom-% excess) expressed as the percentage of the D content (atom-% excess) of body water.)

No. of days on D_2O (*n*)	Mammary gland			Liver		
	Glyceride fatty acids	Phospholipin fatty acids	Cholesterol	Glyceride fatty acids	Phospholipin fatty acids	Cholesterol
1	4·51	3·59	14·26	4·72	4·61	10·8
3	7·00	5·23	31·40	5·88	6·00	24·4
4	8·40	5·60	—	7·80	7·70	29·4
6	20·48	10·13	37·40	12·31	13·90	32·7
12	31·30	16·28	50·00	22·70	21·47	48·4

day onwards (the first three experiments in Table 7), however, the very active fat synthesis must have slackened, since the D content of the glyceride fatty acids increased only slightly between the 24th and 28th days of pregnancy. It is probable that the lack of emptying of the gland retarded fat synthesis in the mammary gland and that the fat which had already accumulated did not participate in active metabolic processes.

In Table 8 are recorded the ^{14}C contents of the fatty acid fractions obtained from the mammary glands of two animals injected with $CH_3{}^{14}COONa$. Qualitatively, the results were the same in all seven experiments, but the amounts of ^{14}C synthesized into the fatty acids of the mammary gland showed much greater variations from animal to animal than in the liver. This might be expected in an organ fulfilling only a temporary function and being greatly influenced by hormonal and other factors.

Table 8. ^{14}C *content of glyceride and phospholipin fatty acids in mammary glands of non-lactating pregnant rabbits injected intravenously with* $CH_3{}^{14}COONa$

(^{14}C content expressed as 1×10^{-5} μc./mg. of fatty acid carbon. In Exp. 1, two equal injections of the labelled acetate were given daily for 4 days; the total ^{14}C dose was 50 μc. (4·3 mg. of acetate). In Exp. 2, two injections, 50 μc. each, were given 6 hr. apart and the animal was killed 20 hr. after the 1st injection.)

	Exp. 1	Exp. 2
Glyceride fatty acids		
Total fatty acids, unfractionated	10·2	42·0
Non-volatile fatty acids:		
Unfractionated	5·1	9·9
Saturated	3·4	7·2
Unsaturated	5·9	10·2
Volatile fatty acids*:		
Water soluble (chiefly butyric)	38·1	184·5
Water insoluble (chiefly caproic)	52·6	136·0
Phospholipin fatty acids	2·91	5·7

* We are indebted to Mr T. H. French of the National Institute for Research in Dairying, University of Reading, for the separation of the volatile acids by steam distillation.

The most important feature of the results shown in Table 8 is the appreciably higher radioactivity of the mixed glyceride fatty acids (volatile + non-volatile) as compared with that of the non-volatile acids. This is accounted for by the much greater isotope content of the short-chain volatile acids (C_4–C_8). It has already been reported (Popják, Folley & French, 1949) that the short-chain volatile acids, which are characteristic components of the milk fat of the herbivores, and which were separated from the mixed glyceride acids of these rabbits, contained 7–18 times more ^{14}C than the non-volatile acids. These results confirm the suggestion of Folley & French (1949) that acetate is utilized for the synthesis of short-chain fatty acids of milk fat. The alternative mechanism for the formation of these fatty acids in milk, by the degradation of long-chain acids suggested by Hilditch (1947), if it occurs, must be of lesser importance.

The non-volatile saturated acids of the mammae (Table 8) contained much less ^{14}C than the unsaturated acids; a notable difference from other organs, e.g. liver, where the reverse occurs. The interpretation of this fact is open to question at present. The results obtained in two animals, in which a comparison could be made with the liver fatty acids, indicated definite synthesis of unsaturated fatty acids in the mammary gland. The low ^{14}C content of the saturated glyceride fatty acids in the breasts might be explained by a relative resting state of the glands before lactation begins, or by assuming that some of the saturated acids (chiefly palmitic) were derived from the blood stream. It should be also borne in mind that the glyceride fatty acid samples were undoubtedly contaminated by connective tissue fat, which cannot be completely removed from the mammary glands. It is not certain what proportion of the fat extracted from the mammae was within glandular secretory cells, or stored in the ducts, or in the interlobular adipose tissue. The presence of the short-chain volatile acids, however, indicates that the material represented primarily fat secreted by the mammae.

DISCUSSION

Cholesterol and fatty acid synthesis in the foetus. The qualitative interpretation of the results presented does not seem to offer serious difficulties. These may be summarized briefly. It has been shown that the foetal liver and the extrahepatic tissues of the foetus are able to synthesize both cholesterol and fatty acids from small molecules and that acetate is a source of carbon in these syntheses just as in the tissues of the adult animal. It appears that no significant amount of cholesterol in the foetus is derived from the preformed maternal compound. The placenta, while able to synthesize cholesterol and fatty acids, also absorbs significant amounts of these substances, or their compounds, from the maternal blood stream (cf. Popják, 1946; Popják & Beeckmans, 1950).

It seems very probable that most of the fatty acids (at least the saturated ones) found in the extrahepatic tissues of the foetus have been synthesized there. The fatty acids in the foetal liver, on the other hand, are derived probably from two sources: (*a*) from synthesis within the organ, and (*b*) from maternal substances transmitted through the placenta. But it is remarkable that the saturated fatty acids do not appear to pass in appreciable quantity from the foetal liver to the rest of the foetal body. It is recalled that in the experiment in which

the animal was given D_2O for 12 days between the 16th and 28th days of pregnancy, the saturated glyceride fatty acids of the foetal carcass had a D content of 50 % and in the foetal liver only 37·6 % of that present in the body water. It may be mentioned in this connexion that, between the 20th and 28th days of pregnancy in the rabbit, the foetal liver becomes extremely fatty, containing occasionally as much as 20 % of neutral fat (Popják, 1946). It is tempting to ascribe this fatty change to infiltration by maternal fat transported across the placenta.

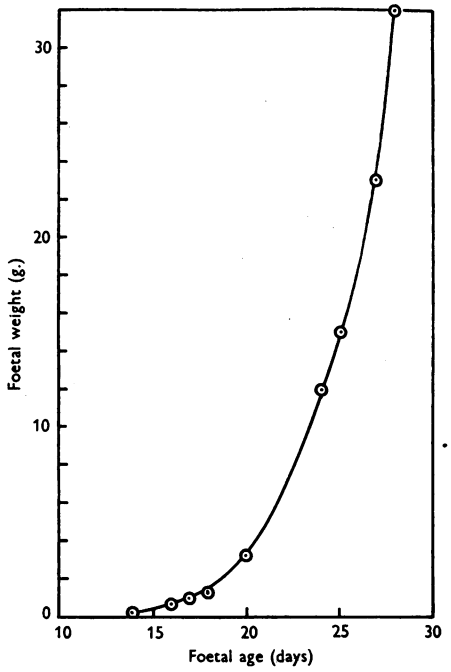

Fig. 2. Growth of rabbit foetus from the data of Boyd (1935) and own observations.

Goldwater & Stetten (1947) concluded that approximately 10·7 % of the foetal cholesterol in the rat was derived from the labelled cholesterol which had been fed to the mother. From the results of this investigation it is inferred that no significant amount of cholesterol in the rabbit foetus could have been derived from maternal sources. This conclusion will be further supported by quantitative assessment of our results. It seems probable that the differences between the results of this investigation and those of Goldwater & Stetten (1947) are due to differences between species. In one experiment in which they gave D_2O to a rat during the whole period of gestation, the D content of the foetal cholesterol was 55 % of that in the body water. In our experiments we found a concentration of about 63 % when the rabbits were given D_2O to drink between the 16th and 23rd or 16th and 28th days of pregnancy.

Calculation of rate of synthesis and rate of degradation of a substance in a growing system from isotope experiments. An important problem in the dynamics of foetal-fat metabolism is to what extent foetal tissues can degrade the fatty substances. To answer this question a quantitative assessment of the results obtained with the aid of D will be necessary.

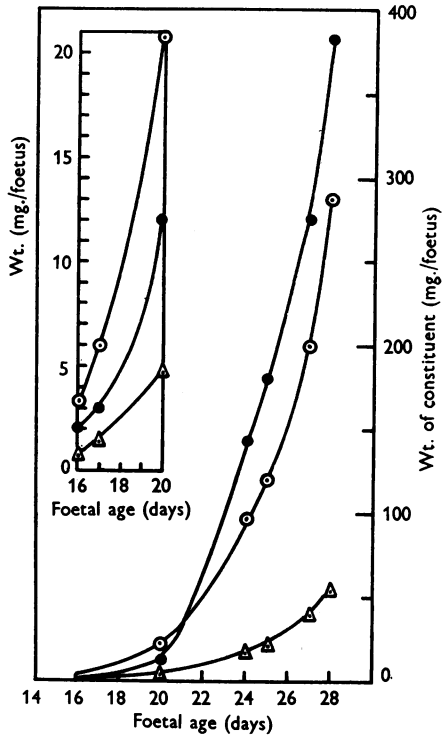

Fig. 3. Accumulation of cholesterol and glyceride, and phospholipin fatty acids in the rabbit foetus. The points give the total amounts of fatty substances present in the foetus between the 16th and 28th days of intrauterine life. The inset on the left shows on a larger scale the quantities present between the 16th and 20th days. Values calculated from data of Boyd (1935) and own observations. Cholesterol, △—△; phospholipin fatty acids, ⊙—⊙; glyceride fatty acids, ●—●.

In the adult animal, in a state of metabolic equilibrium, the rate of synthesis of a compound must be equal to its rate of degradation. In the developing embryo, which shows the phenomenal growth rate illustrated in Fig. 2, this concept of dynamic equilibrium cannot be true; neither can one speak of 'turnover rate' in the manner applied to adult animals; instead two terms, the rate of synthesis and the rate of degradation of a compound must be computed. Fig. 3 shows the accumulation of cholesterol and of fatty acids in the rabbit foetus compiled from the data of Boyd (1935) and our own determination.

The calculations which follow are based on principles similar to those applied by Goldwater & Stetten (1947) to foetal glycogen.

Let dA/dt = the rate of accumulation of substance A in the tissues; db/dt = the rate of synthesis of substance A; $d\alpha/dt$ = the rate of degradation of substance A; then

$$\frac{dA}{dt}=\frac{db}{dt}+\frac{d\alpha}{dt}. \quad (1)$$

Let z = total amount of isotope present in substance A at time t; $A(t)$ = total amount of substance A at time t; then the isotope concentration (i) in the substance at time t is

$$i=\frac{z}{A(t)}. \quad (2)$$

Let i_m = the maximum isotope concentration which can be attained in the compound. If it be assumed that the newly synthesized molecules have an isotope concentration of i_m and the molecules which are being degraded the same isotope concentration (i) as the substance as a whole in the tissues, then the rate of change in z may be written

$$\frac{dz}{dt}=\frac{i_m\,db}{dt}+\frac{i\,d\alpha}{dt}; \quad (3)$$

since

$$i=\frac{z}{A(t)},$$

$$\frac{di}{dt}=\frac{1}{A(t)}\frac{dz}{dt}-\frac{z}{[A(t)]^2}\frac{dA}{dt}; \quad (4)$$

By substituting in (4) the value of dz/dt from (3) and the value of dA/dt from (1), we obtain

$$\frac{di}{dt}=\frac{1}{A(t)}(i_m-i)\frac{db}{dt} \quad \text{or} \quad \frac{db}{dt}=\frac{di}{dt}\frac{A(t)}{i_m-i}. \quad (5)$$

There is no general solution to equation (5), but it may be evaluated empirically by graphical solution of di/dt. It can be shown graphically (Fig. 4) that

$$\frac{di}{dt}=\frac{i_m-i}{\Delta t},$$

and hence substituting in (5)

$$\frac{db}{dt}=\frac{A(t)}{\Delta t}. \quad (6)$$

It should be pointed out that the value of Δt in equation (6), depending as it does on a number of factors, is not the same throughout the growth period of the foetus. For this reason the rates of accumulation, synthesis and degradation have been calculated for the 28th day of pregnancy only, although the experimental data are adequate for similar calculations to be carried out for other foetal ages also.

The data shown in Fig. 3 permit the calculation of dA/dt and the isotope experiments yield db/dt and therefore $d\alpha/dt$ (equation 1) may also be computed. In Figs. 5–8 the continuous curves show the accumulation of the fatty constituents in the foetal tissues. These curves are the same as those shown in Fig. 3 except that they are plotted in reverse, i.e. from the 28th day to the 16th. This was necessary, since this was the time dimension of the isotope experiments with D. The total amount of the substance present in the foetal tissues on any particular day is given in these figures by $(A_{28}-y)$. The left-hand ordinates, y, give the total amount of substance which had accumulated in the foetus between the $(28-n)$th and

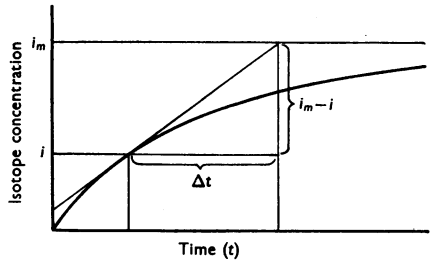

Fig. 4. Graphical evaluation of di/dt, where i = isotope concentration in substance, i_m = maximum isotope concentration attainable in substance. The curve is an assumed isotope concentration-time curve with $i \to i_m$. The procedure is merely to determine the slope of the tangent to this curve.

28th days. For example, the value of y at 22-day foetal age gives the amount of either cholesterol or fatty acids which had accumulated between the 22nd and 28th days of gestation (not the total amount of substance present on the 22nd day, which is given by $(A_{28}-y)$). dA/dt (the rate of accumulation of the substance) on any particular day is given by $(A_{28}-y)/\Delta t'$. The isotope-concentration-time curves (interrupted lines with circles) have been superimposed in Figs. 5–8 on such a scale that the experimentally determined value of i_m (the isotope concentrations found in foetal substances in the 12-day experiment) coincides with the point of A_{28}.

The simple qualitative interpretation of the relationships shown in Figs. 5–8 is that if the points of the isotope-concentration-time curve fall below the curve representing the rate of accumulation of the particular substance, there has been a dilution with substances of lower isotope concentration than i_m. If the points of the isotope-concentration-time curve, on the other hand, are above the curve of accumulation, the substance is being degraded as well as synthesized.

The rates of accumulation (dA/dt), synthesis (db/dt) and destruction ($d\alpha/dt$) of cholesterol and fatty acids have been calculated from the data

shown in Figs. 5–8 for the 28-day-old rabbit foetus. The values are shown in Table 9. The accuracy of these calculations and the deductions drawn from them rest on the correctness of the shape of the

Figs. 5–8. Graphical evaluation of rate of accumulation (dA/dt), synthesis (db/dt) and degradation ($d\alpha/dt$) of fatty substances in foetus with the aid of equations:

$$dA/dt = db/dt + d\alpha/dt \quad \text{and} \quad db/dt = A(t)/\Delta t.$$

(See text p. 555.)

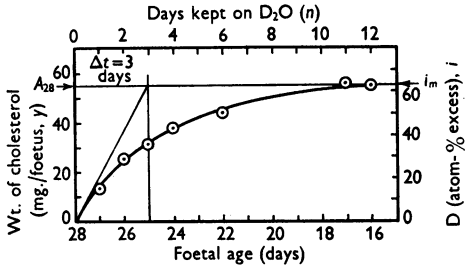

Fig. 5. Foetal carcass cholesterol.

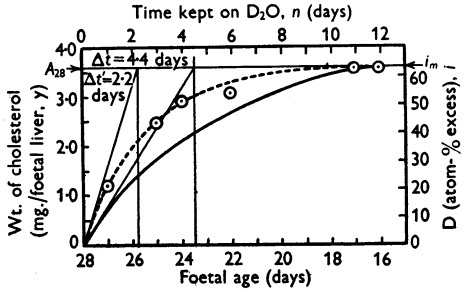

Fig. 6. Foetal liver cholesterol.

curves giving the rates of accumulation of the fatty substances in the foetus; the absolute values of the co-ordinates of these curves are of less importance. Unfortunately, the rates of accumulation cannot be obtained for the same foetuses on which the isotope determinations were carried out, and mean values had to be used from the data of Boyd (1935) and from our own observations for this purpose. The interpretation of the data shown in Table 9 is therefore given with the above reservations.

It appears that the rates of accumulation and synthesis of foetal carcass cholesterol are equal, and therefore there is no destruction of cholesterol by the

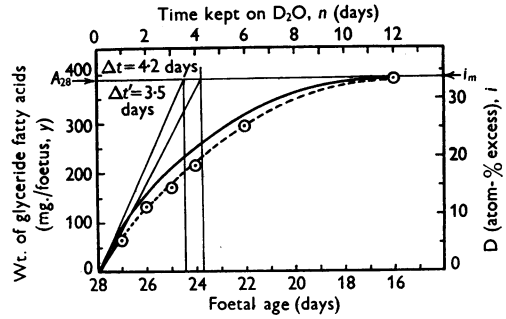

Fig. 7. Foetal carcass glyceride fatty acids.

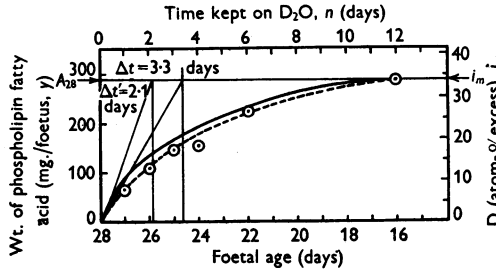

Fig. 8. Foetal carcass phospholipin fatty acids.

extrahepatic foetal tissues. In the foetal liver, however, the values obtained for cholesterol were:

$$db/dt = 1{\cdot}64 \text{ mg./day/liver } (2{\cdot}4 \text{ g.})$$

and

$$d\alpha/dt = -0{\cdot}82 \text{ mg./day/liver.}$$

Thus one-half of what was synthesized on the 28th day was degraded or removed from the foetal liver, resulting in a net accumulation of 0·82 mg. It should be clear from these data that the 55 mg. of cholesterol present in the foetal carcass on the 28th day of gestation could not have been obtained from the

Table 9. *Values of rates of accumulation* (dA/dt), *synthesis* (db/dt) *and degradation* ($d\alpha/dt$) *of cholesterol and fatty acids calculated from data shown in Figs.* 5–8 *for* 28*-day-old rabbit foetuses*

$$dA/dt = db/dt + d\alpha/dt. \quad (1)$$

Source and substance	Wt. of tissues on 28th day (g.)	Total amount of substance present in tissues, A_{28} (mg.)	$\Delta t'$ (days)	Δt (days)	dA/dt (mg./day)	db/dt (mg./day)	$d\alpha/dt$ (mg./day)
Foetal carcass	32	—	—	—	—	—	—
Cholesterol	—	55	3	3	18·3	18·3	0
Glyceride fatty acids	—	389	3·5	4·2	111·2	92·7	+18·5
Phospholipin fatty acids	—	287	2·1	3·3	136·6	87·0	+49·6
Foetal liver	2·4	—	—	—	—	—	—
Cholesterol	—	3·6	4·4	2·2	0·82	1·64	−0·82

foetal liver, even if all the cholesterol synthesized in the latter organ during the entire pregnancy had been transferred to the extrahepatic tissues.

The quantitative interpretation of the results on synthesis of foetal fatty acids is more difficult than in the case of cholesterol. The chief obstacle in the way of precise calculations is the heterogeneity of the fatty acids. It has already been pointed out that in the extrahepatic tissues of the foetus the chief source of both saturated and unsaturated fatty acids is endogenous synthesis. A calculation of rates of synthesis and destruction for the saturated and unsaturated fatty acids separately would be permissible only if the rates of accumulation of the two types of acids in the foetus were known during growth. Such data, however, are not available; only the rates of accumulation of the mixed glyceride and phospholipin fatty acids are known, as shown in Figs. 7 and 8. However, if it be assumed that the ratio of the two types of fatty acids in the foetal carcass is the same throughout the pregnancy and that the newly synthesized mixed glyceride and phospholipin fatty acids contain an average of 33·2 and 34·2 atom-% excess deuterium, respectively, then the figures shown in Table 9 are obtained. The positive values obtained for the rate of destruction ($d\alpha/dt$) of fatty acids indicate that on the 28th day of pregnancy, in addition to synthesis of deuterio-fatty acids, there was a source of non-isotopically labelled fatty acids (or of fatty acids of low isotope content). The most probable source is the maternal plasma. It seems likely that the fatty acids obtained by the foetal carcass from the mother are chiefly the unsaturated acids. This is supported by the observation that when the D concentration of the saturated acids from the foetal carcass is plotted against time of D_2O administration, the shape of the curve obtained approximates the curve showing the rate of accumulation of fatty acids. However, inspection of the figures shown in Table 4 reveals that the D content of the unsaturated glyceride fatty acids in the foetal carcass increased linearly with time, which is quite unusual. This type of curve might result from the placental transfer of maternal unsaturated fatty acids of low isotope content, as already inferred. Naturally these fatty acids would have to traverse the foetal liver first before they can reach the extrahepatic tissues of the foetus. Since the passage of whole phospholipin molecules across the placenta could not be demonstrated (Popják & Beeckmans, 1950), the mechanism of placental transfer of fatty acids remains an open question.

Some differences may exist between foetal liver and the extrahepatic tissues in respect of the mechanism of fatty acid synthesis. This is strongly suggested by the wide discrepancy between the results obtained with the aid of D and of ^{14}C. It will be recalled that in animals given D_2O to drink, the D content of the fatty acids in the foetal carcass was much greater than in the foetal liver. In experiments with labelled acetate, however, the fatty acids in the foetal carcass contained slightly less ^{14}C than in the liver (except for the adipose tissue glyceride fatty acids from the 23-day-old foetus, Table 5) (cf. Tables 2 and 6). If dilution by maternal fat were the sole explanation for the relatively low D content of the foetal-liver fatty acids, one might expect this dilution to be apparent with ^{14}C also. This was not the case. It seems possible that the fatty acid precursors in the extrahepatic tissues of the foetus are more highly oxidized than those in the foetal liver and hence during fatty acid synthesis, which must involve reduction, more D is being introduced in the former than in the latter. It has also been suggested recently by Bloch (1948) in another connexion, that a mechanism of fatty acid synthesis may exist not involving the hydrogen of the body water. This problem is now being investigated.

Reduced rates of, or absence of, degradation of cholesterol and of fatty acids in the foetus appear to be of particular significance. First, as a general biological phenomenon, such an observation may indicate that growth is associated not so much with increased synthesis as with an inhibition of degradation. Rittenberg, Sproul & Shemin (1948) also concluded that the growth of the regenerating liver, after partial hepatectomy, is the result of the inhibition of degradative processes, since the rate of protein synthesis in the regenerating liver was not significantly faster than in normal animals. Secondly, the absence of apparent degradation specifically of fatty acids must mean that the primary energy source in foetal metabolism is carbohydrate. The results of Goldwater & Stetten (1947) are of particular interest in this connexion. These authors have calculated the rate of synthesis and degradation of glycogen in the rat foetus. They found that about 500 mg. of glycogen are synthesized/100 g./day in the 18·75-day-old foetus; of this, however, only about 200 mg. were deposited, the rest being degraded. The amount degraded could have been utilized for energy, or fat synthesis, or both.

SUMMARY

1. The synthesis of cholesterol and fatty acids in the rabbit foetus has been demonstrated by the rapid incorporation of deuterium from the body water into these substances and by the utilization of ^{14}C-labelled acetate for these syntheses.

2. It is concluded (*a*) that all foetal cholesterol is obtained by synthesis within the body of the foetus; (*b*) that while the foetal extrahepatic tissues synthesize cholesterol, they do not degrade it; and (*c*) that the foetal liver synthesizes as well as degrades cholesterol, but the amount degraded is only one-

half of the amount synthesized on the twenty-eighth day of pregnancy.

3. Synthesis of both saturated and unsaturated fatty acids in the foetal liver and extrahepatic tissues (embryonal adipose tissue) has been demonstrated. The fatty acids in the foetal liver contained much less deuterium than those in the extrahepatic tissues; this difference might be due to dilution of fatty acids in the foetal liver by maternal substances. However, when ^{14}C-labelled acetate was given, the fatty acids in the foetal liver contained more ^{14}C than in the foetal carcass. The discrepancy in the results obtained with deuterium and ^{14}C suggests that the fat precursors in the foetal liver and extrahepatic tissues are not identical.

4. The foetal placenta synthesizes all lipids studied, but it also absorbs these from the maternal circulation. The maternal placenta appears metabolically inert, at least towards the end of pregnancy.

5. A method, based on principles developed by Goldwater & Stetten (1947), is presented for the calculation, in isotope experiments, of the rate of accumulation, synthesis and degradation of a substance in a growing system.

6. Since there was no apparent destruction of fat in the foetus, it is inferred that the primary source of energy in the foetus must be carbohydrate and that growth is characterized not so much by increased rate of synthesis as by reduced rate or absence of degradation.

7. The synthesis of cholesterol and fatty acids in the non-lactating mammae of pregnant rabbits has been shown. Evidence for the specific utilization of acetate for the formation of C_4–C_8 acids in the mammae is also presented (see also Popják *et al.* 1949).

We are indebted to Mr D. O'Connor for the mathematical formulation presented in the discussion, to Mr D. Hart for his skilful technical assistance and to Mr F. Crisp for the artificial insemination of the rabbits. The ^{14}C used in this investigation was obtained from the U.S. Atomic Energy Commission; the labelled acetate was synthesized from $Ba^{14}CO_3$ at the Radiochemical Centre, Amersham, Bucks.

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APPENDIX 1

Water Bath suitable for the Maintenance of Temperatures to within $\pm 1{\cdot}5 \times 10^{-3}$ °. Micropipette for the Accurate Delivery of Small Volumes

By G. POPJÁK

Water baths suitable for the determination of deuterium in water samples by the falling-drop method are not available commercially in this country. While such baths have probably been constructed in many laboratories, descriptions are not readily available. The author has been requested from several quarters to describe the bath used in the preceding investigation and which can be constructed by very simple means. No originality is claimed.

Dimensions and materials of bath: 30 × 30 × 50 (height) cm.; galvanized-copper sheet. The bath was made with plate-glass windows at the front and back, and the whole placed in a wooden box. The 2·5 cm. space between the walls of the bath and the wooden box was filled with suitable lagging material. The glass windows were left uncovered.

Thermostat. This was a toluene-Hg thermostat constructed from approximately 5 yards of thin-walled Pyrex