checked by semen collections to insure complete vasectomy and litters 2 and 3 were positively identifiable based on color inheritance as being only from frozen semen.

Polge and Rowson(13) and Bratton *et al.*(12) have shown that storage time is not a critical factor for bovine semen preservation, thereby implying that the same may be true for the rabbit. Detailed experimentation is needed, however, to verify this for the rabbit.

Summary. Three live litters, totaling 13 rabbits, have been produced for the first time from frozen rabbit semen stored 24 hours at −90°C. The diluter used was a modified CUE diluter containing 8% glycerine. Vasectomized males were used to induce ovulation and genetic proof was presented to show that the young could not have come from the vasectomized males.

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Biosynthesis of Cholesterol XI. Biosynthesis of Cholesterol and Fatty Acids in Pregnant Rats.* (27029)

ERWIN SCHWENK AND ERZSEBET JOACHIM (Introduced by G. Pincus)

Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

The formal parallelism of the intrauterine growth of the embryo to tumor growth in the body of the host was the reason why the results obtained in this laboratory in a study of the biosynthesis of cholesterol and fatty acids in tumor bearing animals(1) suggested a similar study of the biosynthesis of cholesterol and fatty acids during pregnancy.

Methods. Virgin female Sprague-Dawley rats weighing about 270 g each were used. They were mated within 6 hours during the same day. All those animals which were allowed to carry to term gave birth to their young 21 days after mating. At the intervals given below for each experiment, a group of rats was injected with 0.5 mc of sodium acetate-1-C^{14} per kg body weight and 2 animals were killed 10, 20, 40, 80 and 240 minutes respectively after the injection. They were then dissected to obtain livers, gastrointestinal tracts, carcasses and fetuses. The tissues, after being weighed were hydrolyzed overnight on a steam bath in 30% KOH. After acidification, cholesterol and fatty acids were extracted with pentane. Purification of cholesterol and counting of samples were carried out as described before(2). Cholesterol determinations in serum were made with the method of Abell et al.(3) using the blood obtained by heart puncture from 4 animals for each determination at 6, 12, 20, 35 and 56 days after mating.

Experiments. Exp. 1: Ten non-mated rats were injected on the mating day with sodium acetate-1-C^{14} as described to serve as controls.

2: Ten pregnant rats were injected 6 days after mating. Because of the impossibility of separating the placenta or fetuses, whole uteri were used.

3: Nine pregnant rats were injected 12 days

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after mating. Because of the smallness of the fetus, placenta plus fetuses were isolated.

4: Ten pregnant rats were injected 20 days after mating. The fetuses were isolated.

4a: Five pregnant rats were injected 21 days after mating and not only the tissues of the mothers were isolated but also the fetuses were dissected and their livers, gastrointestinal tracts and carcasses collected separately. Three of the rats were killed 20 minutes and 2 80 minutes after injection of sodium acetate.

4b: Ten pregnant rats were injected 20 days after mating and not only the tissues of the mothers were isolated but also the fetuses were dissected and their livers, gastrointestinal tracts and carcasses collected. A series of 10 unmated rats were injected at the same time as the pregnant animals.

5: Ten rats were injected 22 days after mating (one day after parturition).

5a: Exp. 5 was repeated because of accidental loss of fatty acids.

6: Ten rats were injected 35 days after mating (14 days after parturition).

6a: Exp. 6 was repeated (using 9 rats) because of accidental loss of fatty acids.

7: Nine rats were injected 52 days after mating (31 days after parturition).

8: For this experiment especially purified cholesterol-H^3 with a specific count of 10×10^6 counts/min/mg was prepared(4). Five mg of it were dissolved in a little ether, mixed with 0.5 cm Tween-20 and the ether evaporated under nitrogen. This solution of cholesterol-H^3 was mixed thoroughly with 9.5 cm of serum from rats' blood, obtained by heart puncture. The serum mixture was incubated at 37°C for 4 hours and should contain cholesterol-H^3 bound as lipoprotein(5). Two-tenths cm of it was injected under slight pentobarbital anesthesia into the femoral vein of each of 10 pregnant rats on the 20th day of pregnancy. Two animals each were killed 10, 20, 40, 80 and 240 minutes after injection and livers, gastrointestinal tracts and fetuses isolated.

9: Cholesterol determinations in serum were made on the pooled blood obtained by heart puncture from 4 animals and taken at 6, 12, 20, 35 and 52 days after mating.

Results and Discussion. The results are presented in Fig. 1-5 and in Table I. Contrary to the findings in tumor bearing animals(1) they show that during pregnancy incorporation of radioactivity from acetate-1-C^{14} into liver cholesterol changes only slightly. This effect may be caused by the age difference between rats at beginning and at end of pregnancy since in experiment 4b control rats and pregnant rats show practically no difference in incorporation of radioactivity into cholesterol of the mother tissues (compare Fig. 3). However incorporation of C^{14} from acetate into cholesterol measured in counts per min per mg does not provide a complete picture. At the twentieth day of pregnancy (Exp. 4) total amount of cholesterol measured in mg (column 7 of Table I) shows a 50% increase over controls. Subsequently through parturition and lactation it falls off slowly to the original level. This increase in amount of liver cholesterol parallels the increase in liver weights in column 2 of Table I and is also in agreement with the serum cholesterol picture as shown in column 13 of Table I. Finally a value about 30% higher than in the normal animal is obtained. The higher serum figures persist through lactation and for 3 weeks after weaning, although at this time total liver cholesterol is at a normal level.

As soon as the young are born, counts in the liver cholesterol of the mothers (Fig. 1) increase considerably. This occurs concomitantly with milk production, and at the time when breast feeding is stopped cholesterol counts surpass by far the original values and those found at the beginning of lactation. Incorporation of C^{14} into the cholesterol of the embryo as shown in Fig. 2 seems to be of no particular significance in the earliest part of pregnancy whereas on the day before birth (Exp. 4) fetal cholesterol has a higher count than the liver cholesterol of the mother. Because the former is diluted with lower counting material from the carcass, Exp. 4a and 4b of Table I were made. Results of Exp. 4b are presented in Fig. 3. The fetal liver cholesterol contains up to 20 times more radioactivity than that from its own parent.

No impressive changes are seen in radioactivity of cholesterol isolated from the gastrointestinal tracts of the mothers during pregnancy and lactation. However the carcasses of

TABLE I. Cholesterol Biosynthesis in Pregnant Rats and in Fetuses.

Expt. No.		Total wt/ animal g 1	Liver wt g 2	G.I. tract wt g 3	Cholesterol mg/g Liver 4	Cholesterol mg/g G.I. tract 5	Cholesterol mg/g Carcass 6	Cholesterol mg in livers of 10 animals 7	Total counts x 10-3 of cholesterol in livers 8	Total counts x 10-3 of cholesterol in whole animals 9	Total counts x 10-3 of cholesterol in fetuses 10	Yield§ in animals 11	Yield§ in fetuses 12	Cholesterol mg in 100 ml serum 13
1	Control	266	10.2	22.5	2.17	1.37	.19	220	304	530	—	398	—	—
2	6 days*	241	9.1	18.9	2.18	1.47	.29	198	302	459	4†	390	3.6	42
3	12 "	281	13.1	27.9	1.92	1.27	.20	252	363	546	13‡	389	95.3	42
4	20 "	325	14.9	32.9	2.15	1.27	.20	320	264	454	540	279	332	78
4a	20 "	335	13.7	26.3	2.24	1.16	—	306	—	—	—	—	—	—
	Fetuses	2.6	.15	.1	2.06	1.78	1.69	—	—	—	—	—	—	—
	Control	200	8.53	23.2	—	—	—	—	—	—	—	—	—	—
4b	20 days	345	14.6	34.1	—	—	—	—	—	—	—	—	—	—
	Fetuses	2.1	.19	.11	—	—	—	—	—	—	—	—	—	—
5	1 day after part.	269	14.9	30.8	1.50	1.17	.62	223	332	536	—	398	—	54
5a	Repeat	286	13.0	33.4	1.65	.75	.31	215	324	491	—	343	—	—
6	14 days after part.	285	18.8	54.6	1.44	.99	.31	271	578	805	—	564	—	—
6a	Repeat**	288	16.0	49.5	1.69	.89	.62	270	778	1,051	—	729	—	—
7	31 days after part.**	257	11.9	27.0	1.94	1.04	.44	230	144	234	—	181	—	66

* Two animals may not have been pregnant.

† Uterus.

** Only 9 rats survived, but figures are corrected for 10 animals.

‡ Placenta plus fetus.

§ Yield $= \frac{10^2 \times \text{Column 9 (or 10)}}{.5 \times \text{Column 1}}$ Factor 0.5 derives from 0.5 mc per kg body wt of rats.

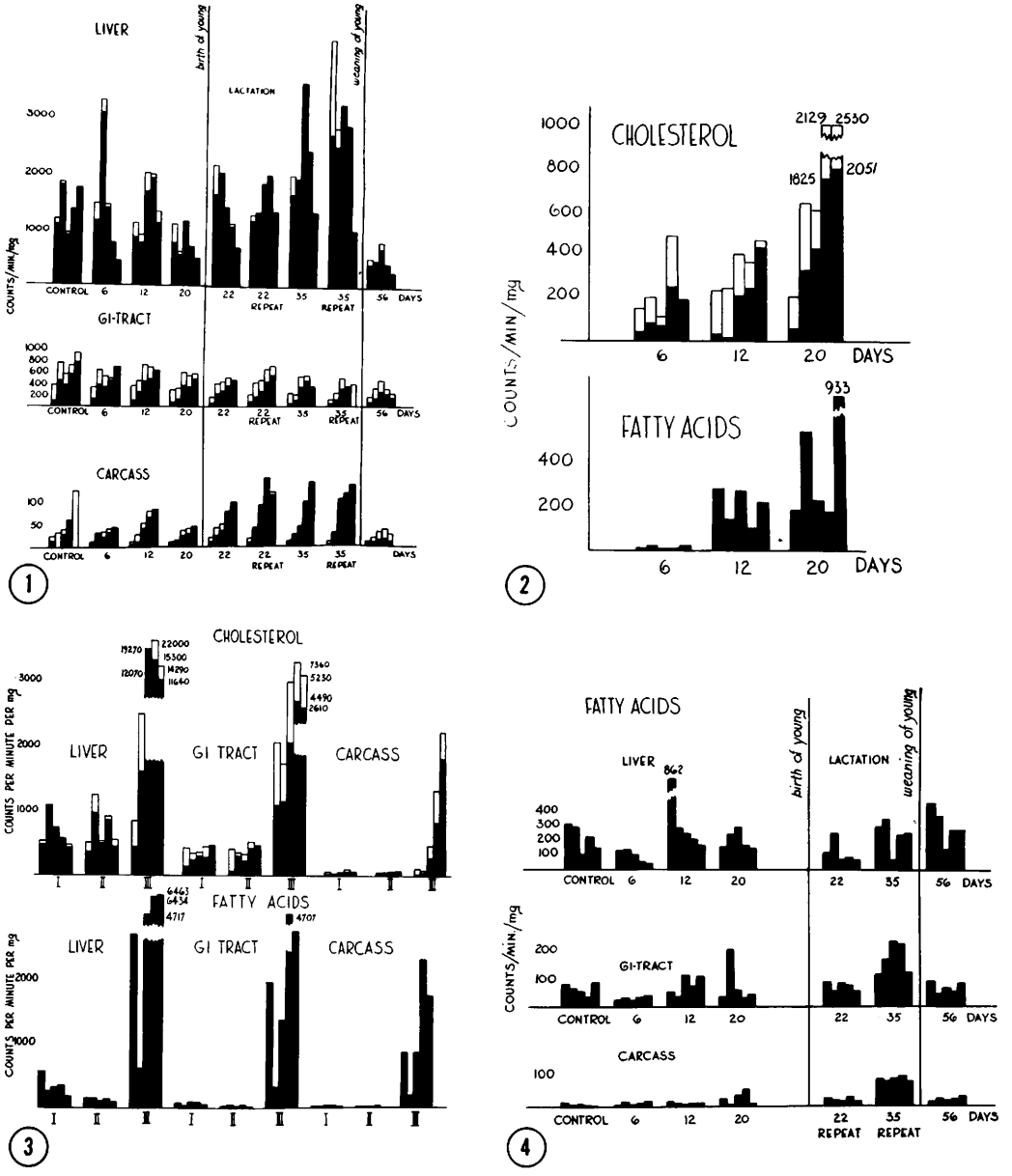

FIG. 1. Exp. 1-7. Cholesterol-C^{14} counts/min/mg in tissues of rats. Each group of bars represents, as in the following charts, a series of rats killed 10, 20, 40, 80 and 240 min. after i.p. injections of sodium acetate-1-C^{14}. Total height of bar gives count in cholesterol from digitonide, black part of bar is count of cholesterol from dibromide.

FIG. 2. Exp. 1-7. Upper row; cholesterol-C^{14} counts/min/mg in uterus (after 6 days), placenta and fetus (after 12 days) and fetus (after 20 days). Lower row is counts/min/mg of corresponding fatty acids.

FIG. 3. Exp. 4b. Cholesterol-C^{14} and fatty acid counts/min/mg in tissues of mother rats and fetuses on 20th day of pregnancy. I are control animals. II are pregnant animals and III are the fetuses.

FIG.4. Exp. 1-7. Fatty acid counts/min/mg in tissues of pregnant rats.

lactating mothers (Fig. 1) contain cholesterol with higher counts than during pregnancy. Again on the day before birth gastrointestinal tract and the carcass cholesterol in the fetus (Fig. 3) has a much higher count than that in the mother's organs.

The counts of fatty acids (Fig. 4) in the organs of the mothers show an increase as the animals approach the end of pregnancy. This increase obtains during lactation and even a month after weaning of the young. In the fetus (Fig. 2 and Fig. 3) at the day before birth, radioactivity in the fatty acids is again very much higher than in the fatty acids of the mother.

During pregnancy there is a weight increase of livers (Column 2) and gastrointestinal tracts (Column 3 Table I). This increase of 30% parallels the increase in total body weights. After birth of the young and during lactation weights of livers and gastrointestinal tracts continue to increase. At the end of lactation the percentage figure calculated on the now lower body weights are about 50% higher than at the beginning of pregnancy. It is interesting to note that the figures for the isolated cholesterol in milligrams per gram of wet tissue from livers and gastrointestinal tracts of the mothers (Column 4 and 5) remain nearly constant throughout pregnancy but then decrease considerably while the young are suckled and until they are weaned. The same figures calculated for the carcasses however (Column 6) increase after birth. All these changes regress after the young are weaned and one month after parturition the figures suggest a slow return to prepregnancy levels. The constancy during pregnancy of the figures for mg cholesterol per gram of wet tissue, suggests that the increases in liver and intestinal weights are not caused by an edema of these organs, which is confirmed by microscopic inspection. No increase in mitotic counts was observed.

It seemed surprising that incorporation of C^{14} from acetate into the cholesterol in fetal tissues appeared so much higher than in corresponding tissues of the mothers. Exp. 8 was therefore carried out to see whether cholesterol passes through the placenta from the mother and is merely stored in the tissues of the fetus. However, only an extremely small

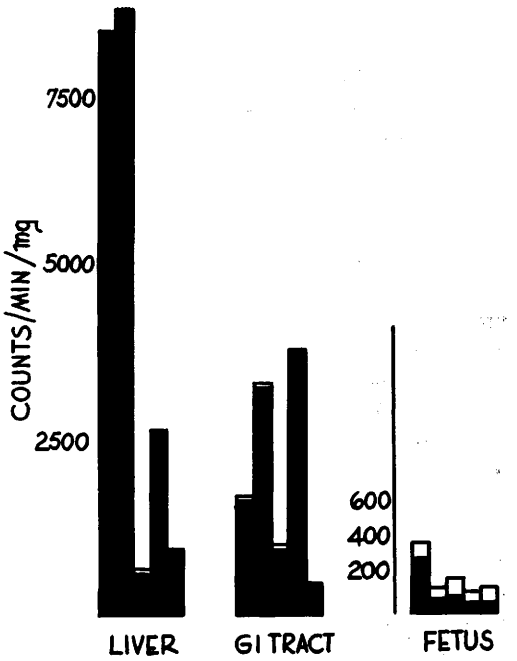

FIG. 5. Exp. 8. Cholestorol-H^3 counts/min/mg in liver and gastrointestinal tracts of pregnant rats and in fetuses on 20th day of pregnancy after intravenous injection of cholesterol-H^3 in rat serum.

part of the radioactive cholesterol passes from the mother's blood into the fetus (Fig. 5). It is clear from this experiment that fetal cholesterol must be synthesized from a water soluble precursor like acetate or mevalonic acid transported by the blood stream through the placenta and then converted by the fetal liver. This raises the question whether the large difference between the activity of the liver cholesterol of mother and fetus could be caused by a higher efficiency in biosynthesis of cholesterol of the fetal liver or by the fact that the fetus does not oxidize cholesterol to bile acids, because it does not digest fat. Inspection of Fig. 3 (Exp. 4b) shows that incorporation of C^{14} into liver cholesterol in control as in pregnant animals occurs at a similar rate. Both values reach their peak at 20 minutes and by 240 minutes are down to 39 and 45% respectively of the peak value. In the fetus liver the highest counts appear somewhat later at 40 to 80 minutes and at 240 minutes are decreased only to 74% of the peak. Rate of decrease is evidently slower in the fetus than in the mothers. As each point

in these charts represents the difference between biosynthesis and degradation of cholesterol up to this time, it is concluded that in the fetus degradation of cholesterol is slower than in the mother while rate of its biosynthesis may be similar for all 3 groups of animals. This difference between biosynthesis of cholesterol and its degradation is mirrored by the "higher counting companions"(2,6), the difference between the counts of digitonin-cholesterol and the counts of pure-cholesterol as represented by the white part of the bars in Fig. 3. They are much larger for the fetus tissues than for the mothers' and suggest a prevalence of biosynthesis over degradation in the fetus.

The highest figures for the counts in the cholesterol of the gastrointestinal tracts in controls, mothers and fetuses are observed at the end of experiment after 240 minutes. Rate of increase is nearly the same for the adult animals but about twice as fast in the fetuses. Figures for carcass cholesterol are again quite similar for control and pregnant rats but are considerably higher in the fetuses, where, at the end of the experiment, they reach 60 times the starting values.

The fact that the embryo in early pregnancy does not produce appreciable amounts of cholesterol is similar to our observation(1) that Yoshida ascites tumor cells do not synthesize cholesterol from acetate. In both instances the same mechanism may be active. The early cells of the embryo may likewise lack the ability to transform acetate into mevalonic acids, which as Popjak(7) has found is converted by tumor cells into cholesterol, while acetate is not. The fetal liver acquires the ability to use acetate only in the final period of pregnancy. The main part of radioactivity evidently passes via the blood stream from the mother into the fetus in the form of acetate because mevalonic acid could not be the precursor of the large amounts of fatty acids produced in the fetus at the end of pregnancy.

The questions of the relation between biosynthesis of lipids in mother and fetus have been studied before by Goldwater et al.(8) for rats and by Popjak(9) for rabbits. Both groups have come to the conclusion that if there is a passage of cholesterol from the mother into the fetus through the placenta, it can only be of minor importance and that cholesterol as well as fatty acids are mainly synthesized in the liver of the fetus. Popjak(10) also found that degradation of cholesterol and fatty acids in the fetus is lower than in the adult so that more of these substances can be accumulated in the tissues of the fetus. The findings here reported support the conclusions of these authors.

Summary. These investigations do not bear out the assumed similarity between the biosynthesis of cholesterol in pregnant rats with the same process in tumor bearing animals. During pregnancy there is apparently no appreciable impairment or other change in rate of biosynthesis in the mother liver although total amounts of cholesterol produced increase with the increase of weights of the livers during pregnancy and more considerably so during lactation, when milk production requires the synthesis of large amounts of fat. It is doubtful whether the early embryonal cell produces any cholesterol from acetate at all or accumulates it. However, at the end of pregnancy the liver of the fetus synthesizes this substance at least at a similar rate as the adult animal, in contrast to the observation that cells of the Yoshida ascites tumor do not make cholesterol from acetate. The degradation of cholesterol in the fetal liver at the day before birth seems to be much less than in the normal controls or the pregnant mothers, thus causing the apparently tremendous incorporation of C^{14} from acetate into fetal liver cholesterol. The experiment shows that only a very minute transfer of this substance from the mother through the placenta takes place. At the day before birth gastrointestinal tracts and carcasses of the fetuses accumulate much higher radioactivities than in the mothers. Synthesis of fatty acids increases during pregnancy in the mothers' tissues and reaches high levels during lactation. At the day before birth the tissues of the fetus show very high levels of radioactivity in the fatty acids.

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Carbohydrate Typing of Mycobacteria* (27030)

EDWARD E. SWEENEY‡ AND GREGORY J. JANN (Introduced by A. J. Salle)

Department of Bacteriology, University of California, Los Angeles

Differentiation of mycobacteria species of medical interest has presented considerable difficulty due to a lack of definitive tests, especially with regard to the "atypical" or "anonymous" acid-fast organisms(1). Biochemical or metabolic testing should be preferred for precise differentiation of microorganisms, but slow-growing mycobacteria are classified at present primarily by cultural morphology and animal pathology. There are several useful biochemical tests, such as Konno's niacin test(2) and the succinamidase test(3), for distinguishing single species or strains of species. Other biochemical tests, e.g. the microcolonial neutral red and cording test(4), aid the grouping of mycobacteria. But no procedures have been reported which appear capable of aiding the typing of all kinds of mycobacteria. Organic acid utilization(5) offers some promise for general speciation, although no reactions were reported positive for the human tubercle bacillus and only one positive reaction was reported for *Mycobacterium avium*.

Carbohydrate utilization testing has not been applied generally to the mycobacteria due to technical difficulties. By adaptation of a procedure reported by Pickett and Nelson(6) for carbohydrate utilization of brucellae, differentiation of mycobacteria strains may be facilitated.

Materials and Methods. Organisms. Mycobacterial strains were inoculated from Lowenstein-Jensen egg medium (stock strains) into 50 ml amounts of TB Broth Base† with 0.5% sterile bovine albumin. No glycerol or other carbohydrate was added. Slow-growing organisms were incubated usually for 7 to 10 days, until just moderately turbid.

Preparation of Inoculum. The bacteria in 25 ml volumes of culture fluid were sedimented by centrifugation at 2,200 revolutions per minute for 3 to 5 minutes; clear supernates were poured off and discarded. Organisms sedimented from 25 ml of culture fluid were resuspended in 0.5 ml of basal medium without agar; 0.1 ml of the suspension was used as inoculum for a single carbohydrate test. The volume of packed wet cells per inoculum was 0.007-0.01 ml.

Basal Test Medium. Basal test medium for 100 tests was composed of: phenol red 4 mg, yeast extract 100 mg, K_2HPO_4 35 mg, agar 1 gm, distilled water 160 ml. The pH was adjusted to 7.0-7.2. Melted medium was dispensed in 1.6 ml quantities in screw-cap vials and autoclaved at 121°C for 15 minutes. Vials were cooled in vertical position; no slant was given the agar.

Carbohydrates. Carbohydrates were pre-

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‡ Present address: Antibiotic Laboratory, Veterans Administration Center, Los Angeles.

† Difco Laboratories, Detroit, Mich.