Metabolism of cholesterol in the tissues and blood of the chick embryo

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ABSTRACT Three artificially inseminated laying White Leghorn hens were given 35–50 μc of cholesterol-4-^{14}C intravenously. Their subsequently produced eggs contained cholesterol-^{14}C-labeled yolks. Some of the fertilized eggs were analyzed for cholesterol content and radioactivity. Other eggs were incubated until hatching. The specific activity of the cholesterol contained in the serum and tissues of newly hatched chicks was determined and compared with that of yolk sac, which was taken as representative of egg yolk cholesterol before its metabolic transfer into the chick embryo. The specific activities of cholesterol in intestine, liver, serum, heart, and skeletal muscle and the whole chick were 95–98% of that in yolk sac, but that of brain cholesterol was only 11% of this value. These results indicate that whereas most of the cholesterol in the chick originated from the egg yolk, cholesterol biosynthesis was active in the brain and provided about 90% of its cholestero content.

Newly hatched chicks were found to be hyperlipemic compared with older chicks and had fatty livers with a high cholesterol content. Desmosterol was found in 9- and 15-day old chick embryos but not in the newly hatched chicks, in which the only sterol was cholesterol.

SUPPLEMENTARY KEY WORDS cholesterol · brain · liver · muscle · yolk sac · biosynthesis · transfer from yolk · hypercholesterolemia · fatty liver · desmosterol · artificial insemination

DESPITE MUCH WORK indicating that the cholesterol of pregnant mammals is transferred into the fetus (1–5), little is known about the origin of the tissue cholesterol of newly hatched birds. By administering cholesterol-4-^{14}C to laying hens, Connor, Osborne, and Marion concluded (6) that most, if not all, of the cholesterol in the egg yolk originated from the blood of the laying hen. However, whether the tissue cholesterol of newly hatched chicks has been transferred from the egg yolk or synthesized in the chick tissues or embryonic membranes remained uncertain.

In 1909, Ellis and Gardner found (7) that eggs and newly hatched chicks contained a similar amount of cholesterol, and suggested that the embryo forms no cholesterol. Twenty years later, Dam determined the cholesterol in both eggs and newborn chicks by digitonin precipitation and reached the same conclusion (8). Failing to observe the incorporation of deuterium from deuterium oxide into cholesterol in incubating eggs, Rittenberg and Schoenheimer reported in 1937 that cholesterol was not synthesized in the egg (9). In studies of the levels of free and esterified cholesterol in the whole egg (yolk and embryo), Tsuji, Brin, and Williams concluded that there was no net cholesterol synthesis in the embryo but that yolk cholesterol is esterified and transferred to embryo (10). Recently, in isolated liver slices from chick embryos Goodridge found a very low rate of cholesterol biosynthesis from acetate (11).

Other investigators have maintained that cholesterol biosynthesis does occur in the chick embryo. In 1931 Needham concluded from the balance experiments of others that a net synthesis of cholesterol can be demonstrated in the developing embryo (12). Bernhard found a significant deuterium content in the cholesterol molecule of the whole embryo after deuterium oxide was injected into the incubated egg (13). Other investigators injected acetate-1-^{14}C into incubating eggs and found radioactive cholesterol in the whole chick embryo (14–17). In 1962, Camerino and Wright injected mevalonic acid-1-^{14}C into incubating eggs on the 6th day of incubation and found that radioactivity was incorporated into the nonsaponifiable fraction of the whole embryo (18).

In view of these divergent results, new studies were designed to provide quantitative information about the origin of chick cholesterol during the entire period of incubation and for the whole chick, the plasma, and in-

dividual tissues. We first labeled egg yolk cholesterol by injecting cholesterol-4-^{14}C intravenously into the inseminated laying hen instead of injecting the radioactive compound directly into incubated egg as an earlier studies. The source of cholesterol of the newly hatched chick embryos incubated from radioactive eggs was then established by comparison of the specific radioactivity of its blood and other tissues with that of yolk sac. We believe that the unutilized cholesterol of the yolk sac is representative of egg yolk cholesterol before its metabolic transfer into the chick. Further information concerning cholesterol precursors in the brain and entire embryo was obtained by determination of the amount of desmosterol in 9-, 15-, and 21-day embryos. The serum cholesterol content of a group of nonradioactive chicks immediately after hatching, and subsequently, was also examined.

MATERIALS AND METHODS

Three White Leghorn hens were given 35–50 μc of cholesterol-4-^{14}C (New England Nuclear Corp., Boston, Mass.) intravenously. This isotope, together with 1 mg of nonlabeled cholesterol in 0.5 ml of ethanol, was mixed with 2.0 ml of chicken plasma and the entire 2.5 ml volume was injected over a 5 min period into the wing vein of each hen. Subsequently, eggs with labeled yolk cholesterol were collected (6). Some of the eggs were analyzed for cholesterol content and radioactivity; others were incubated at 37°C for 21 days until hatching.

The laying hens injected with isotope were artificially inseminated as follows. (This technique was kindly suggested to us by William Marion, Iowa State University, Ames, Iowa.) The feathers surrounding the vent of a healthy White Leghorn male were cut with scissors. The back was then massaged above the thighs and backward along the pubic bones to the vent. Pressure was exerted with the thumb and first two fingers at the base of the vent to stimulate the rooster to protrude the sexual organs. At the same time, the left hand was used to hold a small funnel plugged with wax into which the semen was allowed to run. From 0.5 to 1.0 ml of semen was obtained at each collection. A small glass tube fitted to a piece of rubber tubing was used to draw up 0.1 ml of undiluted semen. This tube was then inserted into the protruded oviduct of a White Leghorn hen and the semen was forced inward by blowing on the other end of the rubber tubing.

The newly hatched chicks were anesthetized with diethyl ether and blood was obtained by cardiac puncture. The chicks were then killed with ether and various organs were collected for analysis. Blood samples from all chicks could not be obtained because of their small size. The serum cholesterol content was determined by the method of Abell, Levy, Brodie, and Kendall (19). For determination of radioactivity, the serum was saponified with alcoholic KOH. The nonsaponifiable residue was extracted with hexane that was evaporated. The residue was then dissolved in 10 ml of scintillation mixture (4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 liter of toluene). The sample was counted in a Packard Tri-Carb liquid scintillation spectrometer with an efficiency of 87%. Results were expressed as specific activity in cpm/mg of cholesterol.

The tissues of different organs were dried under vacuum at 100°C, ground to powder, and extracted with chloroform–methanol 2:1 (20). The aliquots were dried under nitrogen. The cholesterol and cholesterol-4-^{14}C contents of the tissue extracts were determined by the methods described in the previous paragraph for serum (19). Egg yolk cholesterol was extracted and analyzed similarly.

Blood samples from a group of nonradioactive chicks were collected at intervals after hatching and the cholesterol content was determined.

In order to demonstrate that the radioactivity in the tissue extracts was confined to the cholesterol molecule, we chromatographed the tissue lipids on 0.5 mm layers of Silica Gel G in hexane–chloroform–ethyl ether–acetic acid 80:10:10:1. The band of each lipid class (phospholipid, cholesterol, fatty acid, triglyceride, and cholesteryl ester) was scraped off the plate and transferred to a counting vial, and its radioactivity was determined.

That the sterol in the cholesterol and cholesteryl ester bands of tissue extracts actually consisted of cholesterol (5-cholesten-3β-ol) was established as follows. The eluates from thin-layer chromatography were saponified to the free sterol, converted to the trimethylsilyl ether derivatives, and subjected to gas–liquid chromatography on an instrument equipped with a hydrogen flame ionization detector (F&M biomedical gas chromatograph, model 402, Avondale, Penna.). The column was a 1.3 m glass U-tube, 4 mm I.D., packed with 3.8% SE-30 (methyl silicone gum) on 80–100 mesh Diatoport S. Temperatures of column, detector, and flash heater were 230, 250, and 300°C, respectively. Helium was used as carrier gas at a flow rate of 100 ml/min; the inlet pressure was 40 psi. The possibility of partial conversion to cholestanol was eliminated by gas–liquid chromatography of the trifluoroacetates on QF-1 (methyl fluoroalkyl silicone); no cholestanol was found.

The purity of the administered cholesterol-4-^{14}C was verified by thin-layer chromatography as described by Mangold (21). The radioactivity was present in the cholesterol band only.

Because desmosterol has been found in chick embryos and because this sterol reacts like cholesterol with the Liebermann–Burchard color reagent, we determined

the desmosterol content of 9- and 15-day old nonlabeled chick embryos as well as in the labeled newly hatched chicks. The tissues were treated as above and the sterols were determined by gas–liquid chromatography by comparison with pure desmosterol and cholesterol standards.

RESULTS

The transfer of cholesterol-4-^{14}C from three laying hens into the yolk of eggs and later into chicks hatched from radioactive eggs is illustrated in Fig. 1. The specific activity–time curves for cholesterol from eggs and chicks rose sharply to a peak for two of the hens at 5–6 days and for the third at 16 days. The variation in peak specific activity after intravenous cholesterol-4-^{14}C resulted from different egg-laying patterns and has been previously described (6).

Information about the recovery of cholesterol-4-^{14}C in the chick relative to what might have been contained originally in the egg yolk is provided in Table 1. The total radioactivities of the chicks and eggs of each laying hen correlated well and were dependent upon the time of laying. For example, the egg laid on the 5th day after injection of cholesterol-4-^{14}C into hen 37 had 1.93 μc of radioactivity while the chick, incubated from the egg laid on the 6th day contained 1.32 μc. Note that the radioactivities of eggs from the same hen are constantly changing (as per Fig. 1), especially during the initial period after isotope injection.

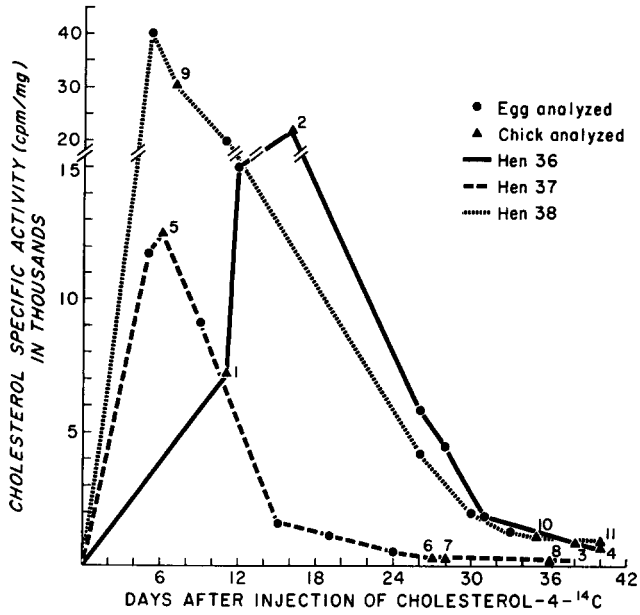

FIG. 1. Specific activity–time curves for cholesterol of eggs and chicks from three laying hens given a single dose of cholesterol-4-^{14}C intravenously. Hens 36, 37, and 38 were given 50, 45, and 35 μc, respectively. Each chick is designated by a number.

Table 2 lists the cholesterol specific activities of various chick tissues. All tissues analyzed from the same chick had similar specific activities except brain, which always had much lower values. Since the radioactivity content of eggs varied a great deal according to the laying date

TABLE 1 COMPARISON OF THE TOTAL AMOUNT OF RADIOACTIVITY IN CHICKS AND IN EGG YOLKS

Days After Injection of Cholesterol-4-^{14}C	Hen 36		Hen 37		Hen 38	
	Chick	Egg	Chick	Egg	Chick	Egg
			μc			
5				1.93		4.01
6			1.32 (5)			
7					4.80 (9)	
9				1.27		
11	0.82 (1)*					2.07
12		2.00				
15				0.23		
16	2.82 (2)					
19				0.20		
24				0.05		
25		0.91				
26						0.54
27			0.03 (6)			
28		0.52	0.03 (7)			
30						0.25
31		0.24				
33						0.14
35					0.09 (10)	
36			0.02 (8)			
38	0.09 (3)					
40	0.07 (4)				0.08 (11)	

* Chick No. in parentheses.

TABLE 2 CHOLESTEROL SPECIFIC ACTIVITY IN INDIVIDUAL CHICK TISSUES

Chick	Yolk Sac	Intestine	Liver	Heart	Skeletal Muscle	Brain	Remainder of Carcass	Whole Chick
				cpm/mg				
1	7,460	7,130	7,060	7,440	7,320	1,090	7,420	6,770
2	22,000	20,900	19,800	18,500	20,800	2,420	20,100	22,400
3	875	934	932	950	938	58	820	876
4	700	720	740	640	736	86	706	775
5	14,900	11,000	14,600	10,000	10,500	1,390	13,200	12,500
6	272	274	304	259	268	34	308	280
7	240	250	243	254	238	32	212	228
8	200	195	136	206	193	22	165	153
9	30,020	32,500	33,700	30,300	28,800	3,230	32,600	31,200
10	1,170	1,100	1,040	1,120	1,140	128	1,170	980
11	850	830	835	825		97	804	792

(Fig. 1), the cholesterol specific activities of the tissues of different chicks derived from the same hen varied considerably. For example, the liver cholesterol specific activities of chicks 1–4 from hen 36 varied from 740 to 19,750 cpm/mg.

Yolk sac cholesterol was considered representative of the original egg yolk cholesterol because it contained unutilized egg yolk. Thus the ratios of cholesterol specific activities of various chick tissues to that of yolk sac were calculated to provide information about the origin of their cholesterol content. This figure ranged from 67.4 to 113% for liver, muscle, heart, intestine, and carcass remnants and from 6.6 to 14.6% for brain. For all 11 chicks, the mean ratio with standard deviation for intestine, liver, heart, skeletal muscle, remainder of carcass, and whole chick were 98.4 ± 2.39%, 97.8 ± 3.81%, 95.4 ± 3.46%, 96.3 ± 3.13%, 96.5 ± 2.75%, and 94.8 ± 3.12%, respectively. In contrast, the mean figure for brain was only 11.3 ± 0.63%.

Serum cholesterol specific activities were obtained from three newly hatched chicks. These values were 757, 220, and 162 cpm/mg of cholesterol for chicks 4, 7, and 8, respectively. The ratios of serum to yolk sac specific activity were 108, 93, and 81%, respectively.

The cholesterol content of individual tissues is expressed in Table 3 as mg/g of dried tissue. The values for liver are remarkably high, at 208 mg/g of tissue. The livers of these newly hatched chicks had a deep yellow coloration and were obviously fatty. We found that the liver cholesterol of 15-day old chick embryo was also high (137 mg/g of dried tissue). The total cholesterol content for each chick was obtained by adding up all of the tissue and yolk sac cholesterol and ranged from 169 to 297 mg. These amounts parallel the range of total cholesterol content of normal egg yolk (170–350 mg).

Table 4 depicts the serum cholesterol concentrations of 10 chicks at hatching and for 17 days subsequently. For the first 3 days the chick is hypercholesterolemic com-

TABLE 3 CHOLESTEROL CONCENTRATION OF INDIVIDUAL TISSUES AND THE WHOLE CARCASS OF NEWLY HATCHED CHICKS

	Individual Tissues							
Chick	Yolk Sac	Intestine	Liver	Heart	Skeletal Muscle	Brain	Remainder of Carcass	Total Cholesterol Content of the Whole Chick
				mg/g				*mg*
1	43.6	26.0	177	31.6	11.9	52.7	14.3	233
2	36.5	34.5	350	18.2	10.9	60.9	14.3	293
3	13.2	27.8	176	24.6	18.8	53.5	14.0	194
4	48.8	26.8	220	17.5	20.8	57.1	13.4	169
5	28.2	34.0	208	17.1	20.2	48.2	12.8	204
6	51.0	12.0	192	20.8	11.3	48.1	8.2	207
7	21.7	24.2	190	17.1	9.4	51.8	9.8	230
8	30.0	24.7	202	16.2	11.4	60.4	12.9	240
9	35.8	26.6	199	29.2	15.2	60.7	11.5	297
10	18.2	24.4	168	16.6	10.9	40.3	13.2	173
11	20.1	29.9	206	24.4	—	57.4	14.5	183
Mean	31.6	26.5	208	21.2	14.1	53.8	12.6	220
SEM	3.84	1.80	5.0	1.64	1.37	1.95	0.61	13.6

TABLE 4 VARIATION OF CHICK SERUM CHOLESTEROL CONCENTRATION AFTER HATCHING

Chick No.	Days After Hatching							
	0	2	3	5	7	9	14	17
	mg/100 ml							
12	286	455	461	226	229	227	123	122
13	489	525	541	311	264	246	140	115
14	369	429	530	288	258	201	156	
15	—	441		296	252	328		
16	427			239		202		
17	429							
18	422							
19	392							
20	458							
21	501							
Mean	419	463	511	272	251	241	140	119

pared to older chickens. The serum cholesterol of the newly hatched (0–3-days old) chick gradually decreased from 419–511 mg/100 ml to 119–140 mg/100 ml at 14–17 days.

The lipid extracts of chick tissues were fractionated into phospholipids, cholesterol, fatty acid, triglycerides, and cholesteryl esters by thin-layer chromatography. The radioactivity of each band was determined by the methods described earlier. The analyses of the liver and yolk sac of chick 1 from hen 36, of the liver and the remainder of the carcass of chick 5 from hen 37, and of the liver, yolk sac, and intestine of chick 9 from hen 38 demonstrated that over 95% of the radioactivity was contained in the cholesterol and cholesteryl ester bands. (The remainder of the count was found at the origin and between the origin and the cholesterol band.) The sterol in the eluates of the cholesterol bands from thin-layer plates was further identified as cholesterol, unaccompanied by other sterols, by gas–liquid chromatography.

No desmosterol was found in any of the tissues of the newly hatched chicks. However, five 9-day old embryos contained considerable amounts of desmosterol: 10.1% (8.7–10.9) of the total sterol in brain and 2.8% (2.6–3.0) in the whole embryo (minus brain). In 15-day old embryos no desmosterol could be detected in two whole embryos (minus brain), and desmosterol represented only 4.6% (4.3, 4.9%) of total brain sterol. No desmosterol was found in 21-day embryos.

DISCUSSION

The design of this experiment was based on several physiological considerations. (*a*) In order to use eggs that would be undisturbed during incubation, we labeled the cholesterol of the egg yolk at the time of its formation in the ovary by injecting cholesterol-4-^{14}C into the laying hen. (*b*) We assumed that the yolk sac probably represented the original status of the egg yolk before its incorporation into chick tissues. (*c*) If cholesterol were synthesized in the embryonic membranes or chick tissues, it would, being unlabeled, dilute the labeled cholesterol derived from egg yolk and thus affect the ratio of the specific activities of cholesterol in the tissues to that of the yolk sac. From our results, we concluded that almost all of the cholesterol contained in newly hatched chick originates from the egg yolk since the specific activities of cholesterol from intestine, liver, heart, serum, carcass, and whole chick were 95–98% of the specific activity of yolk sac cholesterol.

According to calculations from cholesterol specific activity data, 90% of the brain cholesterol was synthesized in situ and did not exchange appreciably with the cholesterol of blood. The metabolism and origin of brain cholesterol has been studied by many investigators. Despite differences in experimental design and species studied, their results (some not quantitative) all point to similar conclusions. Davison, Dobbing, Morgan, and Wright (22) and Kritchevsky and Defendi (23) injected isotopic cholesterol into yolk sac of newly hatched chicks and found only a slight amount of radioactivity in the chick brain analyzed subsequently. Fish, Boyd, and Stokes (17) and Waelsch, Sperry, and Stoyanoff (24) reported the incorporation of acetate-^{14}C and deuterium into brain cholesterol in the chick and rat. Morris and Chaikoff (25) and Clarenburg, Chaikoff, and Morris (26) recovered insignificant amounts of radioactive cholesterol given systemically from the brains of newborn and growing rats and mature rabbits. Our previous report (5) indicated that the brains of fetal guinea pigs and rabbits likewise synthesized most of the brain cholesterol (about 90%).

Further evidence of cholesterol biosynthesis in the brain was provided by the finding of the cholesterol precursor, desmosterol, in 9- and 15-day but not in 21-day embryos. The presence of desmosterol in the chick embryo has been previously reported, especially in the

pioneering observations of Stokes, Fish, and Hickey (14, 15). Weiss, Galli, and Paoletti (27) reported recently that the brain sterols of the chick embryo contained 7.6% desmosterol at 11 days of age and 6.9% at 18 days, results similar to our figures. The disappearance of desmosterol from the brain after 21 days of incubation in our study suggested that during this period active biosynthesis may have been completed.

Several authors (13–18) concluded that cholesterol is biosynthesized in the chick embryo during incubation. That this was at a low level for the entire embryo was suggested by the study of Stokes et al. (14), who found that less than 0.5% of injected acetate-^{14}C was incorporated into cholesterol in 24 hr. This later study was somewhat unphysiologic, in that the acetate-^{14}C was injected into the incubating egg and conceivably might not have readily exchanged with the chick acetate pool. Our study involved individual tissues as well as the whole embryo and, since we used eggs labeled physiologically with cholesterol-4-^{14}C, we did not need to inject the egg while it was incubating. However, our finding that in 9-day embryos 2.8% of the sterols of tissues outside the brain consists of desmosterol suggests some cholesterol biosynthesis in the embryo as a whole.

As proved by both thin-layer and gas–liquid chromatography, the radioactivity of lipid extracts of chick tissues was confined to the cholesterol band. This result further suggested that cholesterol was transferred intact from the blood of the hen through the ovarian membrane into egg yolk and then transferred through embryonic membranes into the chick blood and tissues.

In previous work (5) we had found that the brains of newborn rabbits and guinea pigs contained the highest cholesterol content of all tissues (for example, 39 and 68 mg/g of brain vs. 10 and 9 mg/g of liver). In the newly hatched chick, however, the liver had the highest cholesterol content (208 mg/g of dried liver vs. 54 mg/g of brain). This extremely high concentration of liver cholesterol was probably caused by the direct transference of cholesterol from the cholesterol-rich egg yolk. The reported values of chick brain cholesterol (28, 29) are similar to ours. However, the figure of 13.1 mg/g of liver reported by several investigators (29–31) for the newly hatched chick was much lower than what we obtained even in 15- and 21-day chicks: 137 mg/g at 15 days and 208 mg/g at 21 days. The finding of Goodridge (11) that liver slices from the newly hatched chick synthesized little cholesterol is not surprising in view of the high cholesterol content of the liver; feedback inhibition would be expected to suppress cholesterol biosynthesis in that organ.

The serum cholesterol of the newly hatched chick dropped from 419 to 119 mg/100 ml after 14–17 days. The second value is within the range for older chicks reported by Leveille, Fisher, and Weiss (32). The concentration of cholesterol in the chick tissues drops similarly with time after hatching (33, 34). After hatching, the chick develops its own mechanisms for cholesterol synthesis and excretion and is not being continually challenged with a cholesterol-rich diet from egg yolk.

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