CHOLESTEROL METABOLISM
IN THE
EMBRYONATED HEN EGG

A Thesis presented for the Degree
of
DOCTOR OF PHILOSOPHY
by
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INTRODUCTION

INTRODUCTION

The presence of cholesterol in egg yolk was first shown by Lecanu (1829). Gobley (1846) and Menozzi (1908) examined the cholesterol prepared from this source and found it to be identical with that from gall-stones and bile.

Although other sterols have been found in egg yolk it has been shown that these are present in relatively small amount. Ergosterol separated from 'egg cholesterol' (Windaus and Stange, 1936) was shown by ultraviolet absorption analysis to constitute about 0.1% of the total sterol (Schoenheimer and Dam, 1932; Windaus and Stange, 1936; Skarzynski, 1936). Skarzynski (1936) found the sterol content of the egg to include 3-4% dihydrocholesterol and showed that this proportion did not change after 20 days' incubation. Stokes, Fish and Hickey (1953) employing counter-current distribution methods observed that 95% of the digitonin precipitable material from the egg appeared to consist of cholesterol.

The existence of a high concentration of cholesterol (more than 1%) in egg yolk has aroused considerable interest and many workers have been attracted to problems concerning the metabolism of this substance during embryonic development. Although much of the early work has been vitiated by the use of /

of crude analytical techniques or by failure to take into account the variation in total cholesterol from egg to egg, it is fairly well established that little net change occurs in the cholesterol content of the egg after incubation (Dam, 1928; 1929; Skarzynski, 1936; Stokes, Fish and Hickey, 1953).

Kusui (1929) showed that apart from stray traces cholesterol in the incubated egg is confined to embryo and yolk.

The distribution of total cholesterol between embryo and yolk has interested two groups of workers (Roffo and Azaretti, 1926; Serono, Montezomolo and Balboni, 1936) but in both cases the work involved the analysis of only one egg at each of several stages of incubation. It is therefore not surprising that the conclusions obtained show wide disagreement.

Mueller (1915) first observed that esterified cholesterol which accounts for about 10% of the total cholesterol in the egg at the beginning of incubation increases to over 40% at hatching. Although later workers (Thannhauser and Schaber, 1923; Kusui, 1929; Dam, 1929) confirmed Mueller's (1915) conclusion, little attempt has been made to determine the distribution of ester cholesterol in the incubated egg. Kusui (1929) studied free and esterified cholesterol in both embryo and yolk but his results can be of little significance since the entire investigation was made on a total of only six /

six eggs. It has, however, been shown (Entenman, Lorenz and Chaikoff, 1940) that the liver of the newly hatched chick contains as much as 7% cholesterol (wet weight tissue) of which some 90% is esterified, and that this high concentration of cholesterol diminishes to less than 1% within two weeks following hatching.

Entenman *et al*. (1940) also observed a high concentration of cholesterol in the blood of the newly hatched chick, and a decrease of some 60% during the first two weeks of life. These results are, however, in disagreement with the observations of Zorn and Dalton (1936) who found a hypercholesterolaemia in the blood of the 18 day embryo, a disappearance of this condition at hatching and a rise in blood total cholesterol two days after hatching.

In recent years it has become evident that at least in mammals and more particularly in man, cholesterol is transported in the blood serum in the form of two main lipoprotein fractions which migrate electrophoretically near the α_1 globulins and the β_1 globulins respectively. This work has been collected and reviewed by Cohn (1953). The first work of this kind to be done on birds was reported by McKinley, Oliver, Maw and Common (1953) who found two distinct lipid components in the serum of immature pullets by the application of lipid staining techniques to paper electrophoresis diagrams.

The /

The work reported in this thesis comprised:

(i) An investigation of the changes in free and ester cholesterol in the embryo and the remainder of the egg throughout the course of incubation.

(ii) A study of the lipid changes in the developing chick liver, which seemed of interest in view of the observation by Entenman *et al.* (1940) that the liver of the newly hatched chick is rich in cholesterol esters.

(iii) An attempt to locate the site, or sites, of a cholesterol ester synthesizing system in the incubated egg.

(iv) A re-investigation of the changes in blood cholesterol concentration during embryonic development and in the newly hatched chick.

(v) A study of protein-bound cholesterol in the serum of the late embryo and the newly hatched chick.

SECTION I.

THE DISTRIBUTION OF FREE AND ESTERIFIED CHOLESTEROL IN EMBRYO AND REMAINDER

SECTION I.

THE DISTRIBUTION OF FREE AND ESTERIFIED CHOLESTEROL IN EMBRYO AND REMAINDER

INTRODUCTION.

Lipid solvent extracts of the embryo and the remainder of the egg were used for free and total cholesterol determination by the method of Schoenheimer and Sperry (1934). The modifications suggested by Sperry and Webb (1950) were used, except that the original technique for the neutralisation of the KOH hydrolysate was retained. Proof that the method was applicable to the experimental material used is given in Appendices II and III.

EXPERIMENTAL.

Eggs weighing 55±5g. from the Brown Leghorn strain of the Poultry Research Centre, Edinburgh, were incubated for various periods and then dropped into an acetone-CO_2 freezing mixture for about five minutes. The shell was removed and the egg weighed. The embryo was then dissected out from the frozen egg, transferred to a tared glass dish and weighed, the weight of the remainder being found by difference. The remainder consists of yolk, white, amniotic fluid, allantoic fluid and membranes.

Embryo /

Embryo and remainder were separately homogenized in a Potter-Elvehjem (1936) apparatus with measured volumes of water. Late embryos from which the spare yolk had been removed were first reduced to a crude suspension in an Atomix blendor before being homogenized in the glass-glass apparatus.

Duplicate 1ml. samples of freshly prepared homogenate (occasionally the homogenates were stored overnight in a refrigerator at 4° C) were pipetted into 50ml. volumetric flasks fitted with ground-glass joints, and extracted by refluxing with chloroform-methanol (1:1, v/v) on a steam bath for five minutes. The contents of each flask were cooled and made up to the 50ml. mark with more chloroform-methanol. Suitable volumes (usually 1-3ml.) of the extracts were transferred to 15ml. centrifugal tubes for determination of free and total cholesterol. To avoid the inclusion of protein precipitate in sample extracts, pipettes were fitted with cotton wool filter plugs.

<u>Procedure for Analysis of Free and Total Cholesterol</u>.

<u>Reagents:</u>

Acetone-absolute alcohol (1:1, v/v)
Ether A.R. Grade - peroxide free
Acetone-ether (1:2, v/v)
Digitonin solution, 0.5%. Made by dissolving 500mg. Digitonin (B.D.H. Analar) in 45ml. 95% ethanol and 55ml. distilled water.
Potassium hydroxide solution. Made by dissolving 10g. potassium hydroxide (B.D.H. Analar) in 20ml. water.
Hydrochloric /

Hydrochloric Acid, 5%. Made by diluting 15ml. concentrated hydrochloric acid to 100ml.
Acetic Acid, 100%. B.D.H. Analar.
Acetic Acid, 10%. Made by diluting 10ml. glacial acetic acid to 100ml. with distilled water.
Acetic Anhydride. B.D.H. Analar.
Sulphuric Acid, concentrated. B.D.H. Analar.
Phenolphthalein, 1%.

Precipitation of Free Cholesterol.

The tubes were heated on a water bath and the solvent removed in a gentle air-stream. 2ml. acetone-alcohol (1:1) were added to each residue (containing 0.1-0.2mg. free cholesterol) and the tubes heated momentarily to aid solution. After cooling, 1ml. newly filtered clear digitonin solution was added, followed by 1 drop 10% acetic acid and the contents of the tube thoroughly stirred with a glass rod. The tubes containing the glass rods were placed in tightly sealed preserving jars and left overnight (18 hours) at room temperature. After standing, the tubes were gently stirred again and then centrifuged for 15 minutes at 2,500 r.p.m. The supernatant liquid was carefully decanted without disturbing the precipitate.

After replacing the stirring rods in the appropriate tubes both tube and rod were washed down with 2ml. acetone-ether (1:2) and the precipitate thoroughly stirred up. The tubes were centrifuged at 2,500 r.p.m. for 10 minutes and the supernatant decanted off as before. Two further washings, each of 2ml. ether, were carried out in the same fashion.

After /

After the final washing the tubes together with glass rods were placed in a beaker containing sand to a depth of 3-4cm. and heated in an oven at 110-115°C for 30 minutes. The beaker was then removed from the oven and 1ml. glacial acetic acid pipetted into each tube still in the warm sand. The tubes were stirred to dissolve the precipitate and removed from the sand after not longer than 3 minutes. The tubes were then ready for colour development.

Precipitation of Total Cholesterol.

Residues (containing 0.1-0.2mg. total cholesterol) obtained by the evaporation of chloroform-methanol extracts in centrifuge tubes, were dissolved in 1ml. acetone-alcohol (1:1) and 1 drop potassium hydroxide added. After the alkali drop was dispersed by vigorous stirring the tubes together with rods were placed in a Kilner jar containing sand previously heated to 45°C, and the jar placed in an incubator at 37°C for 30 minutes. On completion of hydrolysis the tubes were removed, allowed to cool and 1ml. acetone-alcohol (1:1) added. 1 drop phenolphthalein indicator was then introduced into each tube and the alkali neutralized by the drop-wise addition of 5% hydrochloric acid. Care was taken to ensure the acid was present in slight excess.

The precipitation and washing were as for free cholesterol with the exception that only one ether wash was included.

Colour /

Colour Development.

The tubes containing 1ml. glacial acetic acid were placed in a water bath at 25° C ± 0.5. The reagent was then made up by adding 1ml. concentrated sulphuric acid slowly and with shaking to 20ml. acetic anhydride, the whole being kept at 0°C in an ice-bath. The reagent was allowed to stand for 10 minutes before use. 2ml. acetic anhydride-sulphuric acid reagent were then added to each tube, the solution thoroughly stirred and the tube returned to the water bath for incubation during 30 minutes in the dark.

The coloured solution was decanted into a 3ml. cuvette and the optical density measured in a Unicam S.P. 350 at a wavelength of 620 millimicrons. Readings were made against a reagent blank.

Two standards of 0.1mg. cholesterol were included in each experimental series to guard against errors arising from day to day variations in the colour reaction. The standards were put through the digitonin procedure since there is a small but perceptible extinction due to the digitonin.

Calibration curves were determined at intervals using amounts of cholesterol varying from 50-300 microgrammes.

RESULTS OF DISTRIBUTION EXPERIMENTS. /

FIG. I.

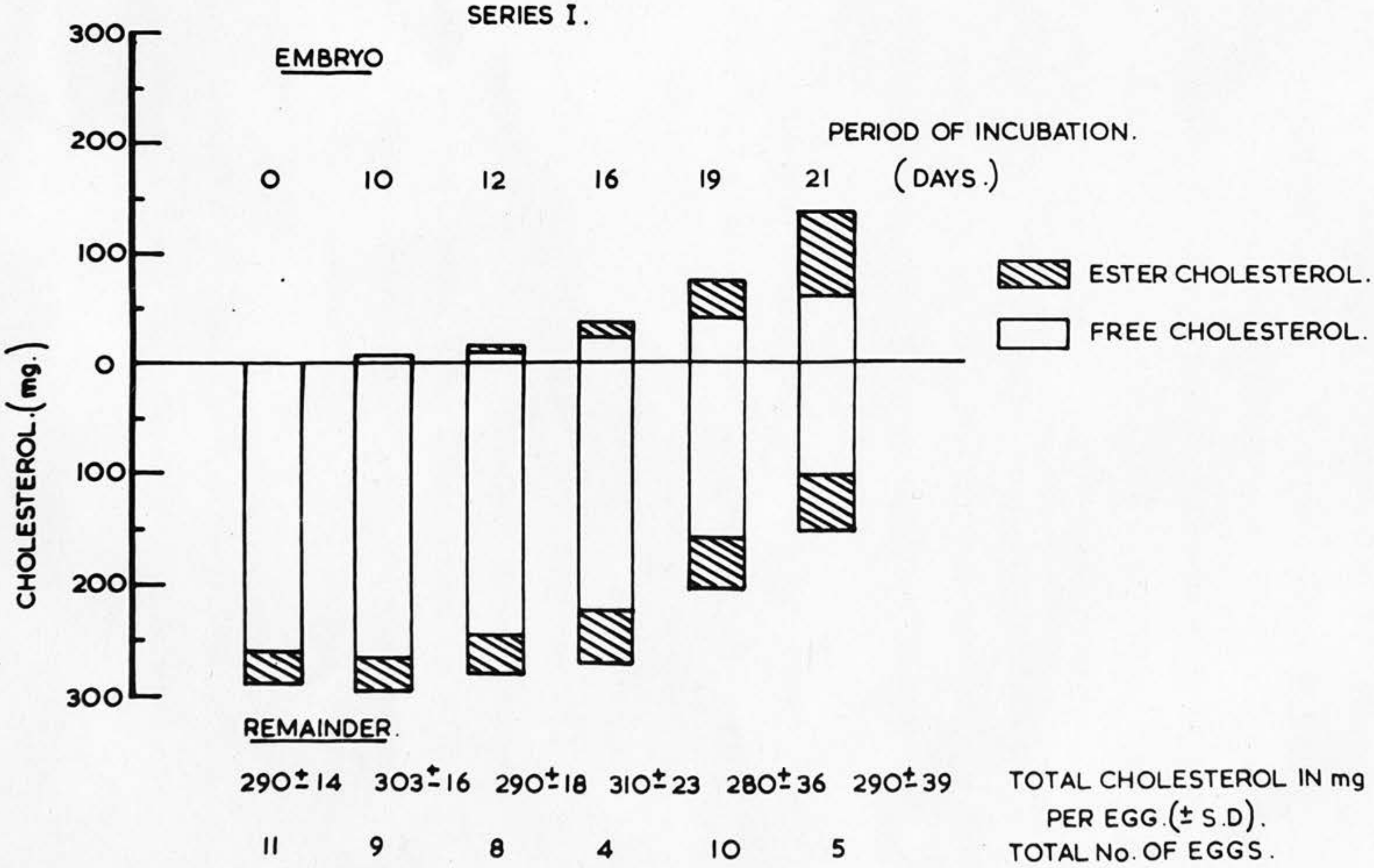
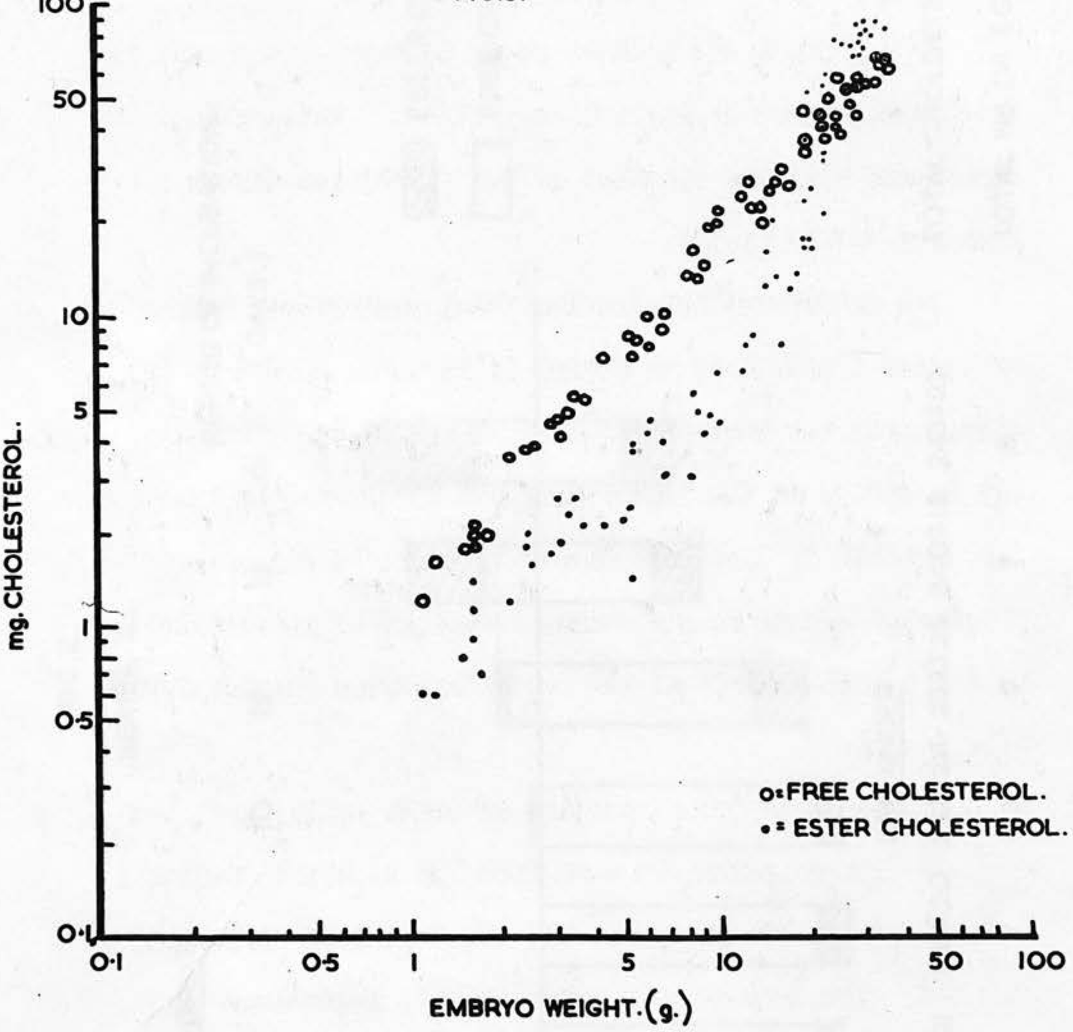

SERIES I.
EMBRYO
REMAINDER.
CHOLESTEROL. (mg.)
300
200
100
0
100
200
300
PERIOD OF INCUBATION.
0
10
12
16
19
21
(DAYS.)
ESTER CHOLESTEROL.
FREE CHOLESTEROL.
290± 14
303± 16
290± 18
310± 23
280± 36
290± 39
TOTAL CHOLESTEROL IN mg PER EGG. (± S.D).
11
9
8
4
10
5
TOTAL No. OF EGGS.

FIG.3.


100
50
10
5
1
0·5
0·1
mg. CHOLESTEROL.
0·1
0·5
1
5
10
50
100
EMBRYO WEIGHT. (g.)
o = FREE CHOLESTEROL.
• = ESTER CHOLESTEROL.

RESULTS OF DISTRIBUTION EXPERIMENTS.

Results from the analysis of 47 eggs between April and October, 1951 (Series I) are shown in Fig. 1, while Fig. 2 summarises determinations made on 56 eggs from February to May, 1952 (Series II). Individual results for both investigations are reported in Appendix IA.

From Figs. 1 and 2 there seems to be no net change in total cholesterol throughout incubation. This is in accordance with the findings of Dam (1929) and Stokes, Fish and Hickey (1953).

The difference between the total cholesterol values in Series I and those in Series II is not a matter of egg size, since the cholesterol content per 50g. unincubated egg is 256mg. in the former case and 230mg. in the latter. The possibility that this disparity might be due to an undetected change in analytical technique is invalidated by the identical plot of the embryo analyses in both Series (see Fig.3).

Increases in the proportion of ester cholesterol in the egg during incubation were from 10% to 43% in Series I and from 6% to 50% in Series II, and show good agreement with the observations of Mueller (1915), Thannhauser and Schaber (1923), Kusui (1929) and Dam (1929). It can be seen from Figs. 1 and 2 that this change in ester cholesterol falls roughly into two parts; a relatively small increase mainly /

mainly in the remainder between 12 and 15 days' incubation, and a large increase, confined to the embryo, which occurs during the last six days of incubation.

Fig. 3 gives all values (Series I and II) for free and esterified cholesterol in the embryo plotted logarithmically against embryo weight and shows more strikingly what can already be concluded from Figs. 1 and 2: free cholesterol increases in direct proportion to the embryo weight throughout the period studied, while ester cholesterol roughly parallels the rise in free cholesterol until an embryo weight of about 16g. is reached (about 16 days' incubation) and then increases ever more rapidly until the time of hatching. This difference in rate of appearance of free and esterified cholesterol in the embryo during the last five days of incubation is further shown by the fact that the proportion of esterified cholesterol in the embryo increases from about 25% of the total at 12 and 15 days of incubation to 60% in the carcass of the newly-hatched chick.

SECTION II.

LIPID CHANGES IN THE LIVER OF THE DEVELOPING CHICK EMBRYO

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LIPID CHANGES IN THE LIVER OF THE DEVELOPING CHICK EMBRYO

INTRODUCTION.

In the previous section it was shown that cholesterol esters in particular accumulate in the embryo towards the end of incubation. Since Entenman, Lorenz and Chaikoff (1940) found the liver of the chick at hatching to have an enormous content of ester cholesterol, the development of this condition in the liver during the course of incubation was studied.

Phospholipid and total fatty acid estimations were included in the analyses.

EXPERIMENTAL.

Livers were dissected out from embryos of 12 to 20 days' incubation and from newly hatched chicks. The gall-bladders were removed and contamination with yolk and embryonic fluids was scrupulously avoided. In the case of embryos younger than 20 days it was necessary to pool several livers for analysis, but whenever possible the organs were analysed individually.

The weighed livers were homogenised with an equal weight of water. Duplicate 1ml. samples of homogenate were extracted /

extracted with chloroform-methanol (1:1, v/v) and made up to 50ml. as described in Section I. Suitable volumes of extract were removed for the determination of phospholipid phosphorus (usually 2-3ml.), total fatty acid (25-30ml.) and free and total cholesterol (0.5-3.0ml.).

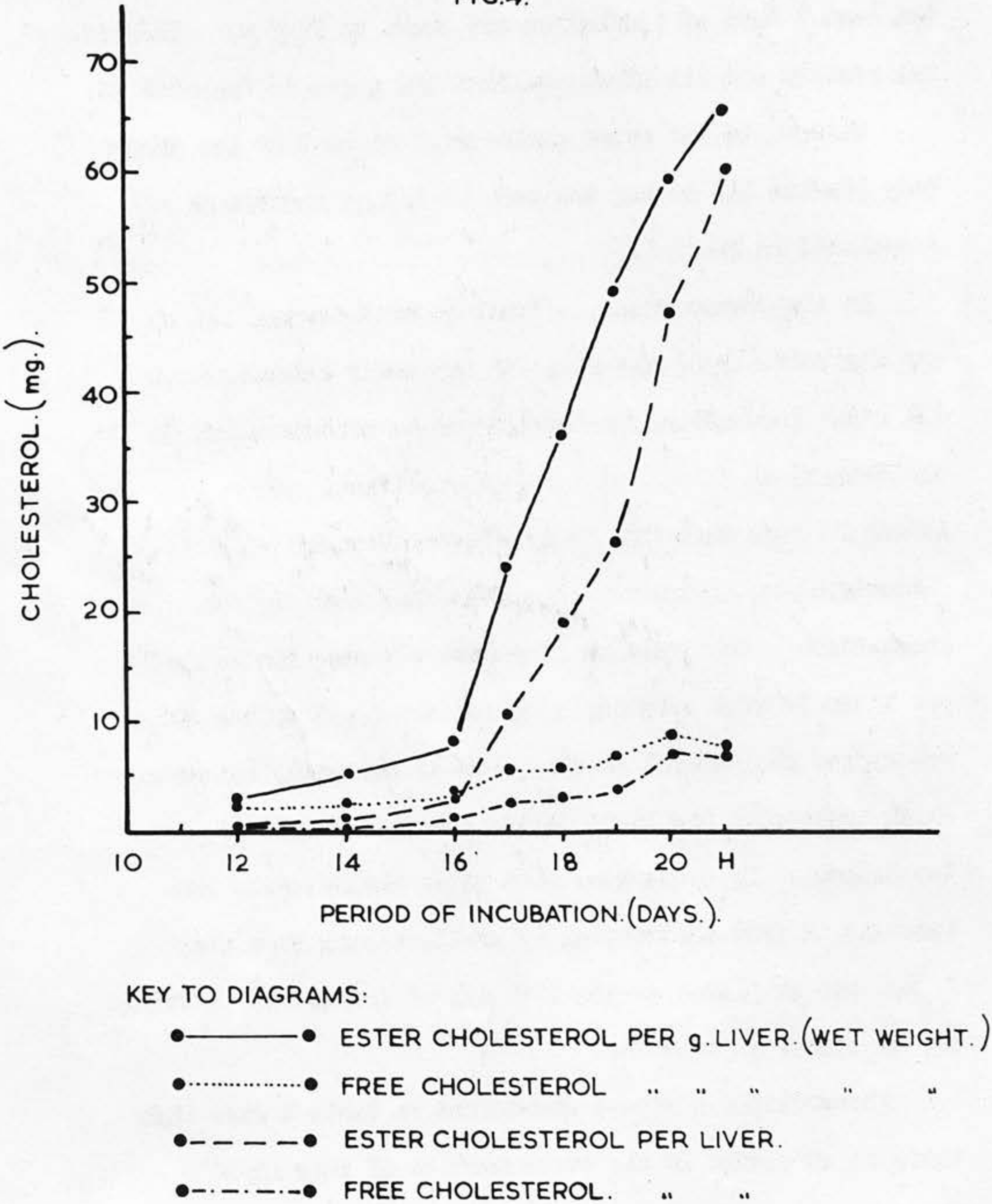
Phospholipid was estimated from phosphorus content of the chloroform-methanol extract using the perchloric acid ashing technique and the molybdenum blue phosphate determination according to Allen (1940). Phospholipid was calculated by multiplying the phosphorus content by 25 (Jamieson and McKinney, 1935). Appendix III includes evidence showing that the phosphorus content of the lipid extract is almost entirely of phospholipid origin.

Analysis of total fatty acid was conducted according to the method of Stoddard and Drury (1929) modified by Man and Gildea (1932). In order to avoid the tedium of the Gooch filtration used by the latter workers the precipitated fatty acids were filtered off on paper as described by Stewart and Hendry (1935).

Free and total cholesterol determinations were made as already described (p. 6).

<u>RESULTS OF LIVER ANALYSES</u>. /

FIG.4.


70
60
50
40
30
20
10
CHOLESTEROL. (mg.).
10
12
14
16
18
20
H
PERIOD OF INCUBATION. (DAYS.).
KEY TO DIAGRAMS:
ESTER CHOLESTEROL PER g. LIVER. (WET WEIGHT.)
FREE CHOLESTEROL " " " " "
ESTER CHOLESTEROL PER LIVER.
FREE CHOLESTEROL. " "

RESULTS OF LIVER ANALYSES.

Changes in concentration and amount of free and esterified cholesterol in the liver of the chick embryo during the last 9 days of incubation are shown in Fig. 4. Individual results for all liver analyses are given in Appendix IB.

Changes in the ester cholesterol content of the embryo body (Series II) during the same period of incubation are summarised in Table 1.

In accordance with the findings of Entenman, Lorenz and Chaikoff (1940) the liver of the newly hatched chick (21 days' incubation) has been found to contain about 7% cholesterol of which some 90% is esterified. From Fig. 4 it can be seen that this high concentration of ester cholesterol mainly accumulates after the 16th day of incubation. The increase in amount of ester cholesterol per liver is even more striking and about 95% of the 60mg. esterified cholesterol in the liver of the newly hatched chick appears in the liver during the last 5 days of incubation. By comparison with ester cholesterol, the increase in free cholesterol is small, rising from about 2.5mg. per g. tissue at the 12th day of incubation to 7.5mg. per g. tissue at hatching.

Phospholipid analyses summarised in Table 2 show that there is no change in the concentration of this lipid throughout the period studied, the values varying between 30.0 and 35.0mg. per g. liver.

Table /

Table 1.

Period of Incubation (days)	Amount of Cholesterol in Esterified Form (mg. per embryo)
12	2.6
15	7.9
19	59.0
21	79.0

Table 2.

Period of Incubation (days)	Phospholipid mg./g. wet liver tissue	Total Fatty Acid m. equiv./g. wet liver tissue
12	33.0	0.096
14	33.0	0.107
16	30.5	0.109
17	35.0	0.168
18	33.3	0.191
19	34.5	0.252
20	33.9	0.275
21	34.0	0.280

Table 2 also includes results for total fatty acid, which show little change between 12 and 16 days' incubation and a rapid rise during the remainder of development. The latter increase broadly parallels that of the ester cholesterol concentration since calculation shows that the cholesterol ester fatty acids account for nearly 90% of the change in total fatty acid.

At hatching, about 60% of the total fatty acid is in the form of cholesterol ester, 30% as phospholipid and 10% as neutral fat.

It is interesting to note that over the period studied the embryo liver changes from the pink colour normally associated with this tissue, at 12 and 14 days' incubation, to a bright yellow during the latter stages of incubation.

The high concentration of esterified cholesterol which develops in the liver during the latter stages of incubation, largely accounts for the accumulation of cholesterol esters in the body of the late embryo and the newly hatched chick.

SECTION III.

CHOLESTEROL ESTERIFICATION IN THE YOLK-SAC MEMBRANE

SECTION III.

CHOLESTEROL ESTERIFICATION IN THE YOLK-SAC MEMBRANE

INTRODUCTION.

Because the increase in cholesterol ester in the yolk precedes that in the embryo (see Fig.2, 15 days) it seemed possible that cholesterol esters might be synthesized in the yolk-sac membrane. The proportion of ester cholesterol was determined in yolk samples free from, and heavily contaminated with, yolk-sac. Since the latter specimens proved rich in ester cholesterol the tissue itself was examined for cholesterol esterifying activity.

Determination of the Proportions of Ester Cholesterol in Yolk and Yolk-Sac Membrane

EXPERIMENTAL AND RESULTS.

Eggs incubated for 10, 14 and 19 days were broken into 0.9% sodium chloride solution and the intact yolk-sac separated from embryo, membranes and fluids. An incision was made in the freed yolk-sac and the yolk was drained off and collected. No attempt was made to remove yolk adhering to yolk-sac membrane. Yolk and yolk-sac membrane were separately homogenized with roughly equal volumes of water

and /

Table 3.

Period of Incubation (days)	Percentage of Total Cholesterol as Ester	
	Yolk	Yolk-sac Membrane (contaminated with yolk)
10	14	33
	11	30
	2	14
14	25	53
	5	30
	2	13
19	13	55
	13	34
	9	37

and analysed for free and total cholesterol by the method previously described (p. 6).

The results are shown in Table 3 from which it can be seen that the proportion of ester cholesterol in the yolk-sac membrane (contaminated with yolk) is 2 to 7 times that in the yolk. Also the quantities of ester cholesterol in the yolk are within the range found in the unincubated egg.

These results suggest that the cholesterol esterifying reaction may be located in the yolk-sac membrane, and that esterification takes place as early as the 10th day of incubation. This experiment does not preclude the possibility that esterification takes place in the yolk immediately in contact with the yolk-sac membrane.

Incubation of Yolk-Sac Membrane Preparations

Attempts were made to demonstrate cholesterol esterification in homogenates of yolk-sac contaminated with yolk and in homogenates of yolk-sac membrane from which as much yolk as possible had been removed by washing with saline. In no case was any cholesterol esterification detected after incubation for even as long as 72 hours. More success, however, attended experiments made by incubating yolk-sac membrane preparations from which much of the ester cholesterol had been removed.

EXPERIMENTAL /

EXPERIMENTAL AND RESULTS.

Yolk-sac membranes were obtained from eggs incubated between 7 and 13 days. The membranes were washed repeatedly with 0.9% sodium chloride solution until no more yolk could be removed and then homogenized with an equal volume of ice-cold 0.9% saline solution in a Potter-Elvehjem (1936) apparatus.

Pooled homogenates from 6 to 8 membranes were transferred to 15ml. tubes and spun for 30 minutes at 2,000 r.p.m. in a refrigerated centrifuge at 2°C. The yolk-like material which formed a semi-solid top layer of the centrifugate was found to contain almost all the ester cholesterol. It was discarded. The small amount of particulate residue was resuspended in the supernatant liquid by vigorous stirring. Duplicate 1ml. samples of the suspension were pipetted into glass-stoppered tubes graduated at 10ml. and the tubes placed in an incubator at 37°C for 72 hours. Shorter incubation times were found to be less effective. Control samples consisting of boiled preparations were included in each series of incubations. The glass apparatus and sodium chloride solutions were sterilised by heat before use.

At the end of incubation acetone-ethanol (1:1) was added to each tube and the tubes heated on a water bath under reflux for 5 minutes. After allowing to cool, the volume in each tube was made up to 10ml. with more acetone-ethanol and /

Table 4.

Period of Incubation (days)	Percentage of Total Cholesterol as Ester Cholesterol	
	Before Incubation	After Incubation
7	0	12
10	15	30
	30	40
	13	21
	27	33
11	7	23
	17	26
	25	33
	22	28
12	7	25
	7	20
	30	39
	22	29
13	2	29
	10	38
	17	31
	18	29
	20	30

Table 5.

Period of Incubation (days)	Percentage of Total Cholesterol as Free Cholesterol	
	Before Incubation	After Incubation
10	39	39
11	45	45
	52	52
12	42	42
	43	43
	52	52
	62	62
13	27	27
	39	39
	42	42

and the tubes centrifuged at 3,000 r.p.m. for 5 minutes. Samples of extract were removed for analysis of free and total cholesterol by the method described previously (p. 6).

Results obtained from a series of preparations before and after incubation are summarised in Table 4. In each experiment the concentration of total cholesterol was found to be unchanged at the end of incubation. The results in Table 4 show that esterification of cholesterol can take place in yolk-sac membrane preparations between 10 and 13 days' incubation and possibly as early as 7 days.

It was not always possible to obtain preparations with a small proportion of ester cholesterol and in general when the initial proportion of ester cholesterol exceeded 30% no esterification took place on incubation. This effect was striking and the "unsuccessful" experiments have therefore been separately grouped in Table 5.

The lack of observations at later stages of incubation arose from the fact that after the 13th day of incubation the yolk-sac membrane became progressively more engorged with yolk. The yolk thus occluded could not be removed with saline and resulted in a failure of the technique for the separation of the ester cholesterol.

These experiments suggest that the increase in cholesterol esters in the "remainder" of the egg may be the result of cholesterol esterifying activity by the yolk-sac membrane.

It /

It is in the liver, however, that most of the esterified cholesterol ultimately appears and the possibility that some esterification takes place in the liver cannot be dismissed. All attempts to demonstrate the formation of cholesterol ester in liver tissue were completely unsuccessful even when preparations low in initial cholesterol ester were made.

An account of these experiments is included in Appendix IV.

SECTION IV.

FREE AND TOTAL CHOLESTEROL IN THE BLOOD OF THE DEVELOPING CHICK EMBRYO

SECTION IV.

FREE AND TOTAL CHOLESTEROL IN THE BLOOD OF THE DEVELOPING CHICK EMBRYO

INTRODUCTION.

The changes in cholesterol distribution during incubation are so striking that it is important to have a clear picture of the state of affairs in the transport system of the blood. Since the figures and results of Entenman, Lorenz and Chaikoff (1940) are at variance with those of Zorn and Dalton (1936) a re-investigation was undertaken.

EXPERIMENTAL.

Eggs incubated for periods between 11 and 21 days were supported end-wise and the shell covering the air-space removed together with the outer membrane. The membrane of the allantois was perforated and the fluid allowed to drain off by tilting the egg. A large blood vessel was then looped over the side of the shell and adhering allantoic fluid dried off with cotton wool. The loop, placed in the mouth of an oxalated specimen tube, was severed and the blood sample collected.

In the case of embryos later than 18 days' incubation the blood was obtained from the carotid artery of the chick. Hatched chicks were anaesthetised with ether before withdrawal of the blood.

Duplicate /

Table 6.

Period of Incubation (days)	Cholesterol mg. per 100ml. blood Free	Ester	Total
11	104	77	181
13	116	76	192
15	113	112	225
17	117	152	269
18	132	183	315
19	145	208	353
20	151	206	357
21	154	211	365

Duplicate 0.2ml. volumes from single and pooled samples were pipetted into glass-stoppered tubes graduated at 7.5ml. and extracted under reflux with acetone-ethanol (1:1) for 5 minutes. The cooled extracts were made up to 7.5ml. with more acetone-ethanol and centrifuged for 5 minutes at 3,000 r.p.m. Samples of the clear supernatant liquid extract were removed for analysis of free and total cholesterol by the method previously described (p.6).

RESULTS OF BLOOD ANALYSES.

The results of free and total cholesterol analyses in chick embryo blood between 11 and 21 days of incubation are summarised in Table 6. Individual values are included in Appendix IC.

From Table 6 it can be seen that following the 13th day of incubation there is a steady increase in blood total cholesterol to a high value at the 19th day, after which time little further change takes place.

About 70% of the increase in total cholesterol which occurs between 13 and 19 days' incubation is accounted for by the rise in ester cholesterol. During this period the proportion of esterified cholesterol changes from 40% of the total cholesterol to 60%.

The results reported here are in agreement with the observations of Entenman, Lorenz and Chaikoff (1940).

SECTION V.

PROTEIN-BOUND CHOLESTEROL IN THE SERUM OF LATE EMBRYO AND NEWLY HATCHED CHICKS

SECTION V.

PROTEIN-BOUND CHOLESTEROL IN THE SERUM OF LATE EMBRYO AND NEWLY HATCHED CHICKS

INTRODUCTION.

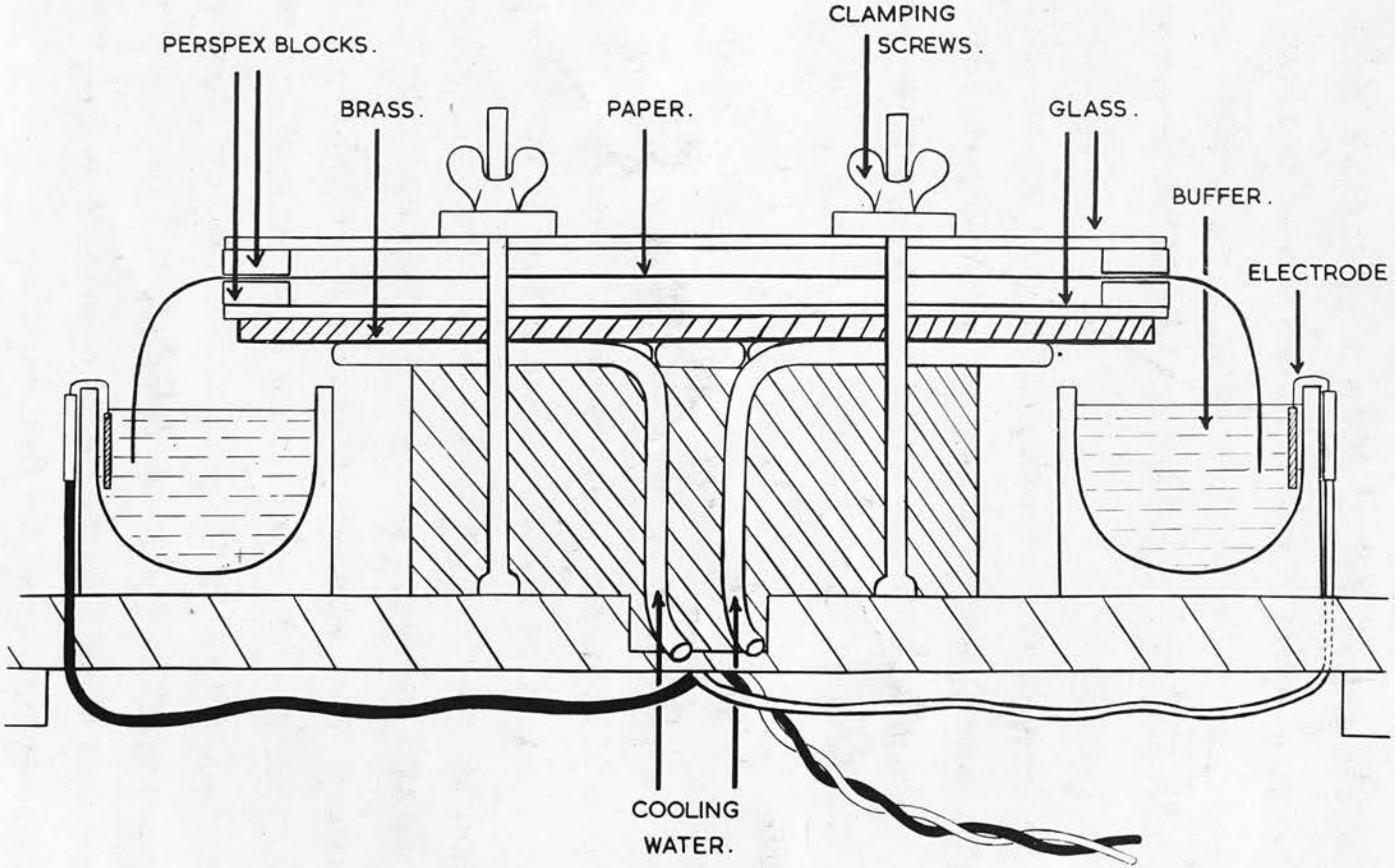
The development of a hypercholesterolaemic condition in the blood of the late chick embryo (Section IV) prompted an investigation into changes of serum protein-bound cholesterol during the latter period of incubation. In view of the additional interest afforded by the observation that the high blood cholesterol concentration in the newly hatched chick diminishes during the first two weeks of life (Entenman, Lorenz and Chaikoff, 1940), it was thought worth-while to extend the study to include the hatched chick.

EXPERIMENTAL.

Embryos were bled from the vitelline vessels and hatched chicks from the carotid. Blood serum samples were stored in a refrigerator at 4°C and used for analysis within 7 days.

The method adopted in this investigation was that of Boyd (1954) and involves zone electrophoresis on filter paper.

FIG. 5.


PERSPEX BLOCKS.
CLAMPING SCREWS.
BRASS.
PAPER.
GLASS.
BUFFER.
ELECTRODE
COOLING WATER.

Apparatus.

The filter paper supporting medium 14" x 5" was suspended between four perspex strips in an air-space formed by two glass sheets 9" x 6" which were clamped to a brass cooling plate. Cooling was provided by the circulation of tap water through a copper coil soldered to the lower surface of the brass plate.

Perspex electrode vessels, fitted with platinum electrodes, were placed one at each end of the clamped plates. The whole apparatus was covered with an inverted glass tank.

The assembly is shown in Fig. 5.

Method.

About 500ml. 0.04M veronal buffer pH 8.6 was run into each electrode vessel and the liquid levels equilibrated by means of a siphon tube placed temporarily between the troughs.

A pencil line was drawn across a piece of Whatman 3 MM filter paper 14" x 5" about 4" from one end. Serum (0.06 or 0.12ml.) was evenly applied to the line previously moistened by spraying with buffer, to within 1/4" from either end. Buffer solution was sprayed on to the paper over the area to be covered by the glass plates, the paper inserted between the glass plates and clamped taut. The ends of the paper were dipped into the buffer solution and a period of 15 minutes was allowed for equilibration.

Electrophoresis was carried out for 8 hours at 12 C with /

with a current of 6 milliamps. at 300 volts.

At the end of the run the paper was dried in warm air, and a narrow strip 9" x ½" removed from the centre of the paper and stained with bromophenol blue to locate the protein fractions. The main portion of the paper was cut into 1cm. sections parallel to the line of origin for individual cholesterol analysis.

Cholesterol Extraction Procedure.

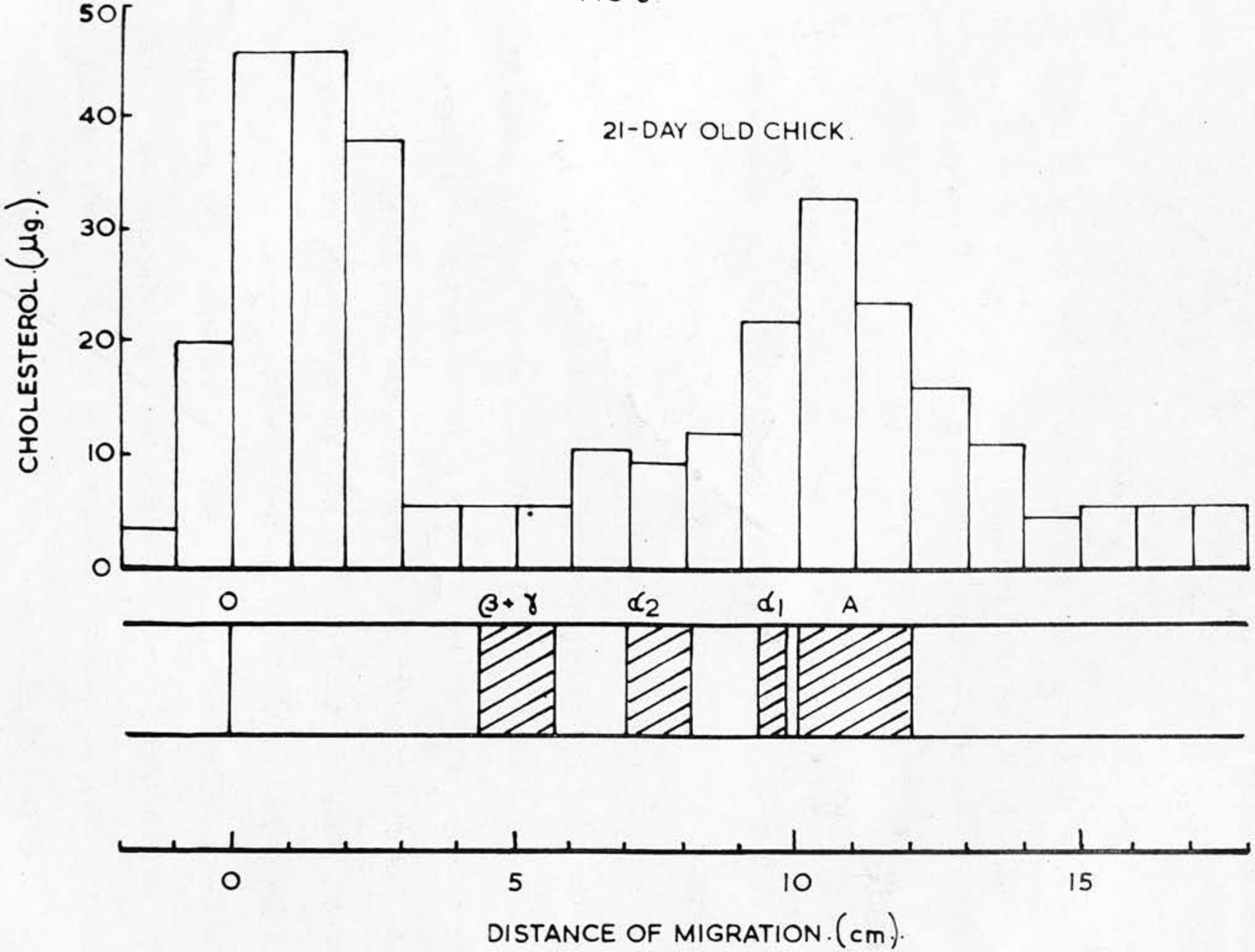
Duplicate transverse segments of filter paper were placed into each centrifuge tube and 7 ml. acetone-ethanol (1:1) added. The tubes were heated on a water-bath to 66°C and maintained at this temperature for 10 minutes. After allowing the tubes to cool the paper strips were removed by forceps and washed with a jet of acetone-ethanol.

Cholesterol Estimation.

The dried residue in each tube was dissolved by the addition of 0.5ml. A.R. glacial acetic acid. 1ml. acetic anhydride-sulphuric acid reagent (prepared as described on p. 9) was added to the acetic acid solution and colour developed by incubation of the tubes in the dark for 30 minutes at 25°C. The coloured solutions were transferred by dry Pasteur pipettes to a 1ml. glass cell with a 2cm. light path and the optical density of each solution read in a Spekker absorptiometer using filter 607, spectrum orange.

Protein /

FIG. 6


50
40
30
20
10
0
CHOLESTEROL. (μg.)
21-DAY OLD CHICK.
O
β + γ
α2
α1
A
0
5
10
15
DISTANCE OF MIGRATION. (cm).

Protein Staining Technique.

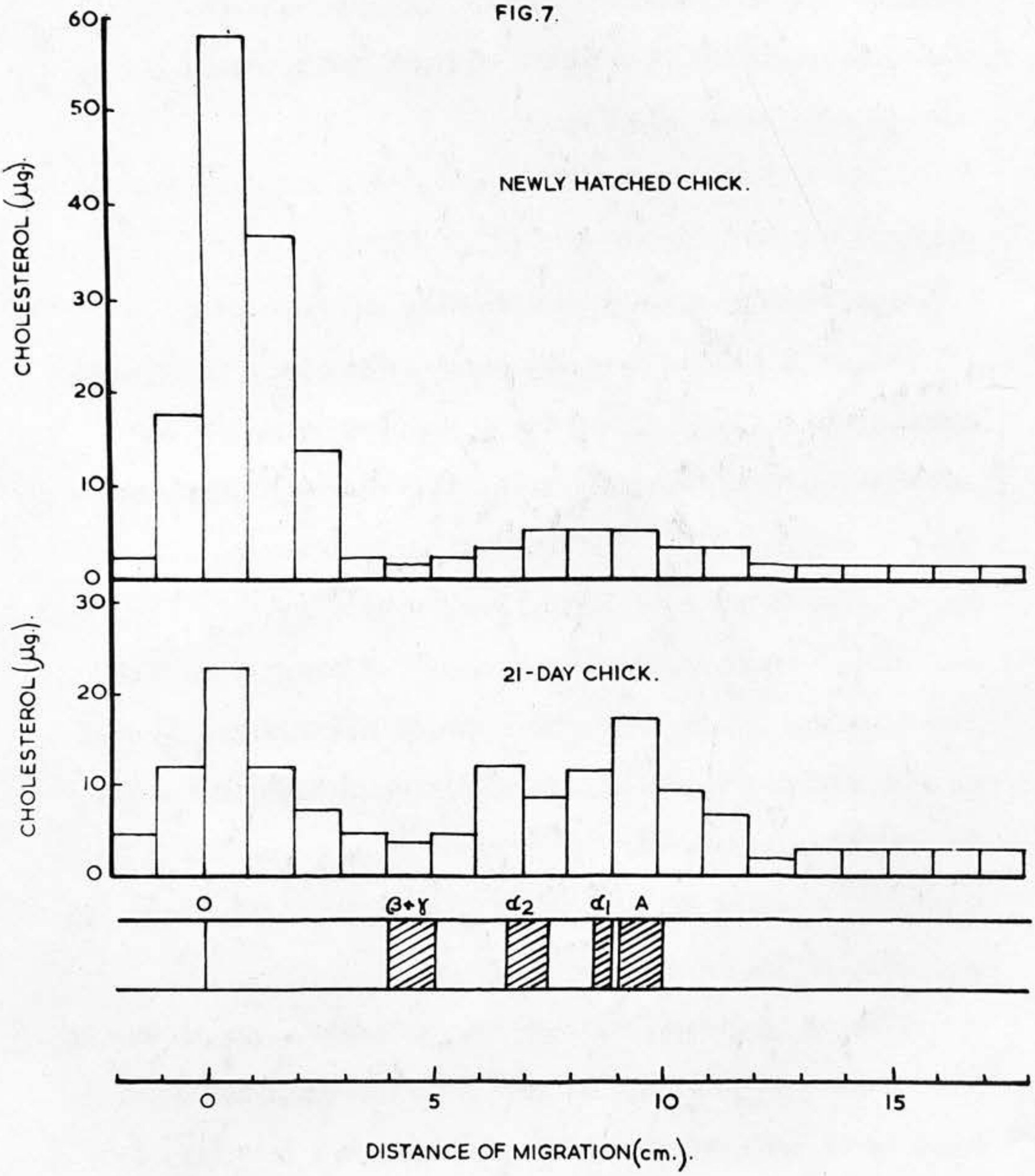
The narrow strip of filter paper retained for protein detection was heated in an oven at 110 °C for 15 minutes, and then allowed to stand for 30 minutes in a 1% solution of bromophenol blue in methanol saturated with mercuric chloride. The paper strip was then washed with 2% acetic acid solution and dried in a warm air-stream.

RESULTS.

Sera from 14- and 17-day embryos, and from chicks 0, 7, 14, 21 and 28 days after hatching, were subjected to electrophoresis on filter paper in the manner described.

Fig. 6 represents the distribution of total cholesterol and the corresponding separation of protein fractions on filter paper following electrophoresis of serum from a 21-day old chick. The protein-stained strip is typical and shows four distinct fractions compared with the five described by Common, McKinley and Maw (1953). These workers, following the precedent established in human studies, named the fractions, albumin, α_1, α_2, β and γ globulins. The technique employed in this investigation has not separated the two slowly moving components (which tended to run together in the work of Common et al., 1953) and the α_1 globulin was sometimes found to be partially obscured by the albumin fraction. There seems to be justification for claiming the separation into /

FIG. 7.



CHOLESTEROL (µg).
60
50
40
30
20
10
0
NEWLY HATCHED CHICK.
CHOLESTEROL (µg.).
30
20
10
0
21-DAY CHICK.
O
β+γ
α2
α1
A
0
5
10
15
DISTANCE OF MIGRATION (cm.).

into albumin, α_1, α_2 and $\beta + \gamma$ globulin fractions. There was no marked deviation from the general pattern over the development period studied. This is in accordance with the observations of Heim and Schechtman (1954) who showed by Tiselius electrophoresis that albumin, α_1, α_2, β and γ globulin fractions which exist in chick embryo serum are also present after hatching.

Fig. 6 shows two distinct cholesterol components which might be termed α lipoprotein (fast-moving component) and β lipoprotein by analogy with studies on human sera, (Kunkel and Slater, 1952; Nikkila, 1953; Boyd, 1954). It should, however, be pointed out that these terms are not intended to imply association of the cholesterol fractions with α_1 and β_1 globulins respectively, as is reported to be the case in human blood serum (Cohn, 1953).

Fig. 7 shows lipoprotein patterns of serum from three newly hatched chicks, and from a 21-day old chick. It will be seen that there is a striking difference between the two. In the younger bird only a very small proportion of the total migrated as α lipoprotein while in the older chick nearly 50% was found in this fraction.

Changes in serum cholesterol concentration are shown in Table 7 from which it can be seen that the total serum cholesterol concentration increased after the 14th day of incubation to a maximum about the time of hatching and declined /

Table 7.

Age of Chick (days from hatching)	Serum Cholesterol mg./100ml.	
	Free	Total
-7	101	206
-4	99	316
0	112	390
7	73	324
14	53	231
21	50	234
28	48	214

Table 8.

Age of Chick (days from hatching)	Cholesterol in mg./100ml. α-lipoprotein	Cholesterol in mg./100ml. β-lipoprotein
-7	13	193
-4	5	311
0	45	345
7	62	262
14	68	163
21	107	127
28	96	118

declined again until about 14 days after hatching.

The quantities of cholesterol present in the two major fractions are shown in Table 8 which summarises the findings at the various ages. These quantities were calculated from the mean total serum cholesterol concentration and the mean percentage of cholesterol in the α and β lipoproteins. The latter values were obtained by summing the area under each cholesterol peak ('background' excluded) and expressing the result as a percentage of the total (Boyd, 1954). (Individual results for serum cholesterol concentration, and the percentage cholesterol in the α and β lipoproteins are shown in Appendix ID.)

The cholesterol in the β lipoprotein rose to a maximum about the time of hatching and decreased rapidly for 2 to 3 weeks while that in the α lipoprotein was almost negligible in the embryo blood and rose steadily from the time of hatching for 2 to 3 weeks.

Since ester cholesterol is known to give a more intense colour than free cholesterol with the Liebermann-Burchard reaction the proportion of ester cholesterol in both α and β lipoproteins was determined. Blood serum from six 21-day old chicks was subjected to Tiselius electrophoresis and the α_1 and β_1 globulin fractions analysed for free and total cholesterol in the manner described previously (p. 6). The results which are shown in Table 9 indicate that there is /

is a slightly greater proportion of ester cholesterol in the α lipoprotein. These values are comparable with observations made on human serum (Kunkel and Slater, 1952).

Table 9.

Proportion of Total Cholesterol as Ester		Cholesterol Concentration in Serum	
α lipoprotein	β lipoprotein	Ester mg./ 100ml.	Total mg./ 100ml.
0.79	0.75	145	190

Since this work was completed Schjeide (1954) has reported a full study of chick serum lipoproteins. He used the ultracentrifuge to separate from the serum of both embryos and hatched chicks, two lipoprotein groups. Schjeide (1954) applied staining techniques to the lipoproteins subjected to ionophoresis and showed that the lipid matter associated with Lipoprotein I scarcely moved from the origin, while the greater part of the lipid from Lipoprotein II migrated immediately behind the albumin. The striking similarity thus shown between Lipoproteins I and II and the β and α lipoproteins (respectively) reported here, is further emphasized by the fact that Schjeide (1954) found most of the embryo plasma lipid to be present in the form of Lipoprotein I, while in the hatched chick the plasma lipid was almost equally /

equally distributed between Lipoproteins I and II. It is important to note however that the latter change took place between the 18th day of incubation and hatching and is consequently much more rapid than the corresponding changes in protein-bound cholesterol noted here.

DISCUSSION

DISCUSSION

Nutrient materials from the yolk are made available to the embryo by absorption through the yolk-sac. This membrane which encloses the yolk grows steadily from the beginning of incubation up to the 15th day, and its highly vascular structure facilitates transportation of yolk constituents into the blood stream and thence to the embryo.

During the course of incubation the cholesterol content of the yolk decreases ever more rapidly and the concomitant appearance of cholesterol in the embryo in equal amount is attended by the development of a hypercholesterolaemia in the embryonic blood. About 75% of the cholesterol in the carcass of the newly hatched chick accumulates in the last 5 days of incubation and the rate of uptake of cholesterol by the embryo during this time amounts to as much as 0.8mg. per g. embryo wet weight per day, while the blood cholesterol concentration rises by some 80%.

The general picture afforded by the changes of cholesterol distribution in the developing egg is therefore compatible with the view that absorption of cholesterol from the yolk takes place. It is important to note, however, that these changes in the egg may, to some extent, be the result of a process involving the synthesis and breakdown of cholesterol. Stokes, Fish and Hickey (1953) were able to demonstrate the continuous uptake of C^{14}carboxyl-labelled

acetate /

acetate into cholesterol during the latter half of incubation and concluded that the apparent rate of conversion of acetate to cholesterol was at all times greater in the embryo than in the yolk-sac or other membranes. These workers however were unable to determine the extent to which cholesterol synthesis and degradation occur.

The latter half of incubation is marked by the appearance of cholesterol esters in the egg and this work has shown the increase in the ester cholesterol content of the 'remainder' is the result of cholesterol esterifying activity in the yolk-sac. In view of the fact that it was not possible to demonstrate cholesterol esterification in the chick liver and since the changes in blood cholesterol concentration largely reflect an increased proportion of esterified cholesterol it is difficult to avoid the conclusion that the yolk-sac membrane is the site of formation of the cholesterol esters which subsequently appear in ever increasing amount in the liver of the embryo. The high concentration of cholesterol esters in the liver after the 16th day of incubation accounts for most of the esterified cholesterol in the carcass of the newly hatched chick.

The finding of a cholesterol esterifying system in the yolk-sac membrane is of considerable interest since this tissue has already been shown to be metabolically active, (Proteinase activity: Borger and Peters, 1933; Goldstein and Ginzburg, 1937: Phosphoprotein breakdown: Sutherland, 1953).

Of particular importance is the study made by Ammon and Schütte (1935) on lipase activity in the yolk-sac in view of the fact that this may furnish the fatty acids necessary for cholesterol esterification. Another possible source of fatty acids for the esterification of cholesterol was revealed by the work of Hevesey, Levi and Rebbe (1938). These workers injected P^{32} labelled phosphate into hen eggs at the beginning of incubation and found that while this isotope was incorporated into phospholipid in the embryo, yolk phospholipid was almost devoid of activity. Hevesey et al. (1938) concluded that the embryo synthesized its own phospholipid and that there was no direct transfer of this material from yolk to embryo. Since it is well known that the phospholipid in yolk-sac and contents decreases during the course of incubation, it is clear that breakdown of phospholipid must take place between the yolk and the embryo. The breakdown of phospholipid might furnish fatty acids in a suitable form for esterification with cholesterol. One is reminded of the lecithinase-B and the cholesterol esterifying enzyme which Le Breton and Pantaleon (1947) claim to be present in blood plasma.

The /

The possibility that endocrine influences might be concerned with the changes which take place in the chick following hatching is suggested by the observation that the "cholesterol fatty liver" condition which is usually resistant to most lipotropic factors (Stamler, Bolene, Harris and Katz, 1950) readily yields to treatment with thyroxine (Stamler, Bolene, Levinson and Dudley, 1950). Despite the fact that the spare yolk with its high concentration of cholesterol is completely absorbed within about 7 days after hatching, the decrease in the cholesterol content of the liver is already considerably advanced at the end of that time (Entenman et al., 1940).

It is worth noting at this stage the interesting parallelism which exists between the changes in the serum of the hatched chick and those occurring in the serum from myxoedematous human patients treated with thyroxine (Malmros and Swahn, 1953). These workers, using filter paper electrophoresis and lipid staining, found the serum from untreated subjects to be rich in β lipoprotein and that after thyroxine administration this fraction decreased together with serum cholesterol concentration. Since two groups of workers (Brody, Funk and Kempster, 1932; Barrott, Fritz, Pringle and Titus, 1938) have demonstrated a marked rise in the basal metabolic rate of the chick during the first two to three weeks of life it is difficult to avoid drawing the complete parallel /

parallel and attributing the changes in cholesterol distribution in the hatched chick to thyroid activity.

SUMMARY.

1. The growing chick embryo subsists on a high cholesterol diet producing a hypercholesterolaemia in the blood and tissues of the late embryo and the newly hatched chick.

2. Cholesterol esters are formed in ever increasing amount during the latter half of incubation and the last five days of incubation are marked by the accumulation of these esters in the liver.

3. The yolk-sac membrane is a site of cholesterol esterifying activity. Cholesterol esterification commences at least as early as the 10th day of incubation.

4. The development of a high concentration of cholesterol in the blood of the late embryo and the decrease following hatching is attended by marked changes in protein-bound cholesterol.

5. Possible mechanisms for the esterification of cholesterol and the possibility of endocrine influence in the newly hatched chick, have been discussed.

APPENDIX IA.

FREE AND ESTERIFIED CHOLESTEROL IN EMBRYO AND REMAINDER

SERIES I.

Unincubated Eggs

No.	*Wt. (g.)	Cholesterol Free (mg.)	 Ester (mg.)	 Total (mg.)
1	55.8	243	54	297
2	54.5	262	29	291
3	55.1	257	27	284
4	57.3	271	39	310
5	58.6	251	20	271
6	60.0	270	40	310
7	57.5	281	24	305
8	57.8	262	19	281
9	55.7	264	20	284
10	57.2	260	21	281
11	54.5	255	20	275
Mean (±S.D.)	56.7	261	29	290 ± 14

* Including shell.

SERIES I.

10 Days' Incubation

No.	Wt. (g.)	Embryo Cholesterol Free (mg.)	Embryo Cholesterol Ester (mg.)	Wt. (g.)	Remainder Cholesterol Free (mg.)	Remainder Cholesterol Ester (mg.)	Total Chol. (mg.)
1	3.1	4.6	2.6	41.3	262	48	317
2	1.2	1.6	0.6	45.5	284	15	301
3	1.5	-	-	45.4	299	22	323
4	2.5	3.8	1.6	43.1	287	25	317
5	2.4	3.7	1.8	43.1	262	33	301
6	3.7	5.3	2.1	49.7	247	39	293
7	3.1	4.2	1.8	45.7	247	40	293
8	3.3	4.8	2.3	46.1	274	28	309
9	3.1	4.7	1.8	45.9	240	24	271
Mean (±S.D.)	2.7	4.1	1.8	45.1	267	30	303 ± 16

SERIES I.

12 Days' Incubation

No.	Wt. (g.)	Embryo Cholesterol Free (mg.)	Embryo Cholesterol Ester (mg.)	Wt. (g.)	Remainder Cholesterol Free (mg.)	Remainder Cholesterol Ester (mg.)	Total Chol. (mg.)
1	5.2	8.1	3.6	39.4	235	61	307
2	5.3	8.1	3.8	42.8	247	49	308
3	6.0	7.8	4.2	39.7	260	38	309
4	5.2	7.4	1.4	39.3	242	26	277
5	6.7	9.0	3.9	34.8	238	36	287
6	8.6	12.9	4.9	37.1	252	24	294
7	9.0	14.5	4.2	33.0	229	16	264
8	6.8	10.2	3.1	36.8	245	15	273
Mean (±S.D.)	6.6	9.8	3.6	37.9	244	33	290 ± 18

SERIES I.

16 Days' Incubation

		Embryo			Remainder		Total
No.	Wt.	Cholesterol		Wt.	Cholesterol		Chol.
	(g.)	Free (mg.)	Ester (mg.)	(g.)	Free (mg.)	Ester (mg.)	(mg.)
1	14.4	19.9	16.1	27.1	203	49	288
2	15.0	25.0	20.0	30.5	229	68	342
3	14.2	21.9	12.6	29.3	216	51	302
4	13.2	22.1	8.4	33.6	248	28	307
Mean (±S.D.)	14.2	22.2	14.3	30.1	224	49	310 ± 23

19 Days' Incubation

		Embryo	
No.	Wt.	Cholesterol	
	(g.)	Free (mg.)	Ester (mg.)
1	21.7	43	31
2	22.2	41	33
3	19.5	36	23
4	21.9	39	25
5	23.8	40	40
Mean	21.8	40	30

19 Days' Incubation

		Embryo			Remainder		Total
No.	(Wt. g.)	Cholesterol		Wt.	Cholesterol		Chol.
		Free (mg.)	Ester (mg.)	(g.)	Free (mg.)	Ester (mg.)	(mg.)
1	19.5	37	24	25.8	182	61	304
2	22.0	41	21	22.0	197	49	308
3	18.9	34	17	26.8	202	45	298
4	19.1	34	16	21.3	179	57	286
5	19.5	33	17	28.0	168	48	266
6	17.1	26	12	25.7	182	16	236
7	21.9	37	54	19.6	126	60	277
8	25.9	52	52	14.6	109	26	239
9	28.8	53	53	12.7	111	19	236
10	28.5	53	70	16.7	141	82	346
Mean (±S.D.)	22.1	40	34	21.3	160	46	280 ± 36

SERIES I.

21 Days' Incubation
(Hatching)

No.	Embryo			Remainder			Total Chol. (mg.)
	Wt. (g.)	Cholesterol		Wt. (g.)	Cholesterol		
		Free (mg.)	Ester (mg.)		Free (mg.)	Ester (mg.)	
1	33.4	63	65	11.9	121	66	315
2	35.3	65	82	6.7	70	35	252
3	35.3	61	66	12.6	132	66	325
4	31.3	67	78	9.9	130	40	315
5	28.0	54	84	7.0	64	42	244
Mean (±S.D.)	32.7	62	75	9.6	103	50	290±39

SERIES II.

Unincubated Eggs

No.	*Wt. (g.)	Cholesterol (Whole Egg) Free (mg.)	Ester (mg.)	Total (mg.)
1	54.2	229	20	249
2	54.5	213	7	220
3	54.5	238	24	262
4	54.8	237	11	248
5	53.3	237	8	245
6	53.8	248	8	256
7	52.0	213	8	221
8	55.5	233	5	238
9	54.0	207	18	225
10	53.8	237	18	255
11	50.0	230	25	255
12	55.7	269	28	297
Mean (± S.D.)	53.8	233	15	248 ± 21

* Including shell.

SERIES II.

6 Days' Incubation

No.	*Wt. (g.)	Cholesterol (Whole Egg)		
		Free (mg.)	Ester (mg.)	Total (mg.)
1	42.7	216	23	239
2	44.8	227	18	245
3	46.0	245	13	258
4	43.5	215	17	232
5	44.7	221	23	244
6	44.0	252	30	282
7	45.0	246	36	282
Mean(± S.D.)	44.4	232	23	255 ± 20

* Excluding shell.

SERIES II.

10 Days' Incubation

		Embryo			Remainder		Total
No.	Wt. (g.)	Cholesterol Free (mg.)	Cholesterol Ester (mg.)	Wt. (g.)	Cholesterol Free (mg.)	Cholesterol Ester (mg.)	Chol. (mg.)
1	1.6	2.1	1.4	40.2	232	34	270
2	1.6	2.0	1.1	41.5	221	9	233
3	1.6	1.9	0.9	39.1	220	21	244
4	1.5	1.8	0.8	39.5	207	18	228
5	1.1	1.2	0.6	37.2	198	14	214
6	1.7	2.0	0.7	45.3	237	17	257
7	2.2			37.8	227	30	257
8	1.6			47.4	278	33	311
9	2.0			38.5	242	7	249
10	2.0			39.5	228	19	247
11	1.5			45.0	227	26	253
12	1.6			47.1	242	31	273
13	2.0			45.8	212	22	234
Mean	*1.5	*1.8	*0.9	41.8	229	22	252 ± 24

* Mean of 6 values.

Nos. 7-13 inclusive: Embryo Cholesterol Included in Total.

SERIES II.

12 Days' Incubation

No.	Wt. (g.)	Embryo Cholesterol	
		Free (mg.)	Ester (mg.)
1	5.1	8.6	2.4
2	4.3	7.5	2.1
3	3.5	5.5	2.6
4	2.9	4.4	1.7
5	6.0	9.9	4.6
6	5.0	7.8	2.2
Mean	4.5	7.3	2.6

SERIES II.

15 Days' Incubation

No.	Embryo Wt. (g.)	Embryo Cholesterol Free (mg.)	 Ester (mg.)	Remainder Wt. (g.)	Remainder Cholesterol Free (mg.)	 Ester (mg.)	Total Chol. (mg.)
1	15.5	27.1	13.2	27.0	165	38	243
2	18.0	35.1	13.5	26.6	178	37	264
3	9.4	18.8	4.7	33.1	206	25	255
4	10.0	19.5	4.5	36.0	242	32	298
5	12.0	24.0	6.6	31.3	191	44	266
6	16.0	29.6	8.0	23.0	149	35	222
7	10.0	21.5	6.5	27.0	187	51	266
8	12.5	28.1	7.9	25.7	141	46	223
9	8.2	13.2	6.3	34.8	170	44	234
Mean (± S.D.)	12.4	24.1	7.9	29.4	181	39	252± 25

SERIES II.

19 Days' Incubation

No.	Embryo Wt. (g.)	Embryo Cholesterol Free (mg.)	 Ester (mg.)	Remainder Wt. (g.)	Remainder Cholesterol Free (mg.)	 Ester (mg.)	Total Chol. (mg.)
1	29.0	51	70	9.0	69	36	226
2	25.0	38	54	10.0	100	31	223
3	29.0	56	74	10.0	87	52	269
4	19.0	46	50	14.3	87	42	225
5	21.5	41	43	13.3	133	41	258
6	23.0	50	58	11.0	92	36	236
7	27.8	45	66	10.0	102	31	244
Mean (± S.D.)	24.9	47	59	11.1	96	39	240± 18

SERIES II.

21 Days' Incubation

No.	Embryo			Remainder			Total
	Wt.	Cholesterol		Wt.	Cholesterol		Chol.
	(g.)	Free (mg.)	Ester (mg.)	(g.)	Free (mg.)	Ester (mg.)	(mg.)
1	27.0	48	72	9.0	100	40	260
2	30.0	53	85	7.0	80	65	283
3	24.0	43	74	6.0	79	44	240
4	24.5	53	73	7.0	69	43	238
5	29.0	56	78	6.0	52	34	220
6	29.5	55	79	3.5	40	39	213
7	30.0	53	80	7.0	79	34	246
8	33.0	56	87	7.0	68	34	245
Mean (± S.D.)	28.4	52	79	6.5	71	42	243± 22

APPENDIX IB.

LIVER ANALYSES

LIVER ANALYSES

12 Days' Incubation

No. of livers		Wt. per liver (g.)	Cholesterol mg. per g. tissue Free	Cholesterol mg. per g. tissue Ester	Phospholipid mg./g. tissue	Total Fatty Acid m. equiv./g. tissue
6		0.06	2.4	2.0	33.0	-
7		0.07	2.0	2.4	32.5	0.0971
6		0.08	2.5	3.1	33.7	0.0955
19	Mean	0.07	2.3	2.5	33.0	0.0963

14 Days' Incubation

4		0.18	2.7	5.9	33.0	0.110
5		0.18	2.6	4.8	32.5	0.107
7		0.15	2.6	5.4	33.2	0.102
16	Mean	0.17	2.6	5.3	33.0	0.107

16 Days' Incubation

7		0.34	3.9	9.1	33.0	0.116
3		0.23	2.6	6.3	25.0	0.0934
10	Mean	0.31	3.5	8.3	30.5	0.109

17 Days' Incubation

4		0.45	5.7	20.3	35.0	0.164
4		0.45	5.0	28.0	35.0	0.173
8	Mean	0.45	5.4	24.2	35.0	0.168

18 Days' Incubation

4		0.50	6.4	35.0	32.5	0.191
3		0.55	4.6	37.5	34.4	0.192
7	Mean	0.52	5.6	36.1	33.3	0.191

19 Days' Incubation /

19 Days' Incubation

No. of livers		Wt. per liver (g.)	Cholesterol mg. per g. tissue		Phospholipid mg./g. tissue	Total Fatty Acid m. equiv. / g. tissue
			Free	Ester		
3		0.51	9.5	43.0	35.0	0.220
3		0.52	5.6	44.9	33.2	0.244
3		0.58	6.8	55.3	35.0	0.277
2		0.51	5.6	49.9	34.5	0.268
2		0.54	5.6	52.9	35.0	0.260
13	Mean	0.53	6.8	48.8	34.5	0.252

21 Days' Incubation

No. of livers		Wt. per liver (g.)	Free	Ester	Phospholipid	Total Fatty Acid
1		0.8	11.0	51.5	35.0	0.246
1		0.8	10.3	33.2	35.5	0.246
1		0.8	7.5	64.5	37.5	0.298
1		0.79	6.4	59.6	32.0	0.290
1		0.83	7.5	47.5	35.5	0.256
1		0.87	9.0	62.5	33.2	0.281
1		0.67	9.0	83.3	32.5	0.315
1		0.75	9.0	69.5	30.0	0.266
8	Mean	0.79	8.7	59.0	33.9	0.275

21 Days' Incubation

No. of livers		Wt. per liver (g.)	Free	Ester	Phospholipid	Total Fatty Acid
1		0.95	9.0	51.0	33.7	0.238
1		1.00	8.3	47.2	32.0	0.216
1		0.69	7.2	54.3	30.0	0.235
1		1.15	7.7	68.9	35.0	0.300
1		0.86	7.5	73.3	35.0	0.296
1		0.73	7.2	67.8	35.0	0.285
4		0.95	7.4	72.6	35.0	0.309
10	Mean	0.92	7.6	65.3	34.0	0.280

APPENDIX IC.

BLOOD CHOLESTEROL VALUES

BLOOD CHOLESTEROL VALUES

Period of Incubation (days)		No. of Eggs/ Determination		Cholesterol in mg./100ml.		
				Free	Ester	Total
11		6		106	68	174
"		6		106	83	189
"		6		101	79	180
	Total	18	Mean	104	77	181
13		6		113	74	187
"		4		119	71	190
"		4		119	83	202
	Total	14	Mean	116	76	192
15		3		125	101	226
"		3		94	126	220
"		3		110	124	234
"		3		115	101	216
"		3		119	111	230
	Total	15	Mean	113	112	225
17		2		119	148	267
"		2		115	155	270
"		2		112	136	248
"		2		123	166	289
	Total	8	Mean	117	152	269
18		1		120	170	290
"		1		135	225	360
"		1		112	203	315
"		1		112	203	315
"		3		147	161	308
"		1		130	173	303
"		1		137	195	332
	Total	9	Mean	132	183	315
19		1		151	211	360
"		1		144	202	346
"		1		158	217	375
"		1		137	194	332
"		1		144	216	360
"		1		137	209	346
	Total	6	Mean	145	208	353

20 /

BLOOD CHOLESTEROL VALUES

Period of Incubation (days)		No. of Eggs/ Determination		Cholesterol in mg./100ml.		
				Free	Ester	Total
20		1		144	209	353
"		1		137	195	332
"		1		151	195	346
"		1		166	223	389
"		1		158	187	345
"		1		151	224	375
	Total	6	Mean	151	206	357
21		1		160	235	395
"		1		140	208	348
"		1		165	210	375
"		1		176	222	398
"		3		147	201	348
"		1		144	214	358
"		1		158	209	369
	Total	9	Mean	154	211	365

APPENDIX ID.

BLOOD SERUM CHOLESTEROL VALUES

BLOOD SERUM CHOLESTEROL VALUES

Period of Incubation (days)		No. of Eggs/ Determination		Cholesterol in mg./100ml.	
				Free	Total
14		6		99	208
"		6		104	204
	Total	12	Mean	102	206
17		4		105	317
"		4		-	308
"		4		87	295
"		4		106	345
	Total	16	Mean	99	316

Age of Hatched Chick (days)		No. of Chicks/ Determination		Cholesterol in mg./100ml.	
				Free	Total
0		4		68	308
"		3		127	402
"		4		144	483
"		3		102	382
"		3		130	321
"		3		104	458
"		3		111	375
	Total	23	Mean	112	390
7		3		87	348
"		3		78	278
"		2		90	392
"		2		38	269
"		2		63	342
	Total	12	Mean	73	324
14		2		67	234
"		2		69	260
"		2		42	204
"		2		-	214
"		2		36	241
	Total	10	Mean	54	231

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BLOOD SERUM CHOLESTEROL VALUES

Age of Hatched Chick (days)		No. of Chicks/ Determination		Cholesterol in mg./100ml.	
				Free	Total
21		1		65	259
"		1		42	265
"		1		40	174
"		1		-	185
"		1		55	287
	Total	5	Mean	51	234
28		2		57	227
"		2		49	216
"		4		44	188
"		1		49	237
"		1		49	237
"		1		41	244
	Total	11	Mean	48	214

APPENDIX ID.
(continued)

PROPORTION OF SERUM CHOLESTEROL IN THE FORM OF LIPOPROTEIN

PROPORTION OF SERUM CHOLESTEROL IN THE FORM OF αLIPOPROTEIN

Period of Incubation (days)	No. of Eggs/ Determination	%age Total Cholesterol in form of α lipoprotein
14	6	5.0
"	6	8.6
	Total 12	Mean 6.8
17	4	3.3
"	4	0.0
"	4	1.4
"	4	1.8
	Total 16	Mean 1.6

Age of Hatched Chick (days)	No. of Chicks/ Determination	%age Total Cholesterol in form of α lipoprotein
0	3	21.8
"	3	0.0
"	3	14.7
"	4	11.8
"	3	5.9
"	3	6.8
"	3	27.0
"	4	11.4
"	3	5.1
	Total 29	Mean 11.6
7	2	29.3
"	2	18.3
"	3	15.7
"	2	16.4
"	2	27.0
"	3	23.4
"	2	18.0
"	2	5.1
	Total 18	Mean 19.2

14 /

PROPORTION OF SERUM CHOLESTEROL IN THE FORM OF α LIPOPROTEIN

Age of Hatched Chick (days)	No. of Chicks/ Determination	%age Total Cholesterol in form of α lipoprotein
14	2	14.7
"	2	31.3
"	2	37.2
"	2	22.6
"	2	42.5
Total	10	Mean 29.7
21	1	46.8
"	1	33.3
"	1	51.1
"	1	59.3
"	1	41.8
"	1	42.4
Total	6	Mean 45.8
28	2	50.9
"	2	45.8
"	4	40.3
"	1	36.8
"	1	40.7
"	1	61.8
Total	11	Mean 44.9

APPENDIX II.

EXAMINATION OF DIGITONIN PRECIPITABLE MATERIAL FROM LIPID EXTRACTS OF EGG CONTENTS

EXAMINATION OF DIGITONIN PRECIPITABLE MATERIAL FROM LIPID EXTRACTS OF EGG CONTENTS

The method of cholesterol determination employed throughout this work depends on application of the Liebermann-Burchard reaction to the digitonide precipitate. Since it is known that a number of sterols give colours with this reaction a study was made of the digitonin precipitable sterol content of the egg.

(a) Separation of Sterol Material from the Digitonide Precipitate.

The entire contents of six 5-day eggs (total weight 322g.) were homogenized in an Atomix blendor and one-sixth part (by weight) of the homogenate extracted by refluxing with 1 litre chloroform-methanol (1:1, v/v) for 2 hours. The extract was filtered by suction on filter paper (lipid free) and the precipitate washed with two 100ml. volumes hot chloroform-methanol. The combined filtrate and washings were taken to dryness on a steam-bath and the residue dissolved in 200ml. 10% (w/v) potassium hydroxide in 95% ethanol. After refluxing for 4 hours the solution was cooled and made slightly acid (phenolphthalein indicator) with concentrated hydrochloric acid. Precipitation with digitonin was carried out according to Windaus' (1910) procedure. After centrifuging for 15 minutes at 2,000 r.p.m. the /

the supernatant was decanted off and the residue thoroughly stirred with 100ml. acetone-ether (1:2, v/v). The process of centrifugation and decantation was repeated. Two further washings each of 100ml. ether were given in the same manner.

After heating in an oven at 110°C for 30 minutes (to remove ether) the digitonide was treated with 30ml. pyridine on a steam-bath for 1 hour and then 500ml. ether added. The precipitate which formed was submitted to a second regeneration with pyridine. The combined ether extracts were taken to dryness under reduced pressure on a water-bath and the residue re-extracted with five 20ml. portions of ether. The brownish-white crystalline material remaining after evaporation of the ether was dried for 24 hours at 110 C under reduced pressure - yield, 235mg.; m.p. 141-145 C.

(b) Isolation of Cholesterol.

120mg. crude sterol were dissolved in 3ml. ether and the solution maintained at 0°C. Bromine was added drop-wise until in slight excess. After 30 minutes at 0 C, 3ml. glacial acetic acid were added and crystals which formed during the next 10 minutes were filtered, washed with 5ml. cold methanol and sucked dry. The white crystalline material was stirred for 30 minutes with 2g. zinc dust and 3ml. glacial acetic /

acetic acid. The mixture was then extracted four times with 15ml. ether and the combined ether extracts washed twice with 10ml. 10% sodium hydroxide solution and twice with 20ml. water. The ether solution was taken to dryness under nitrogen and the residue recrystallised from methanol to which a few drops ethyl acetate had been added. The crystalline product was filtered, washed with cold methanol and dried at 110 °C under vacuum for 12 hours. Yield, 95mg.; m.p. 149 °C; mixed m.p. with pure cholesterol, 149 °C.

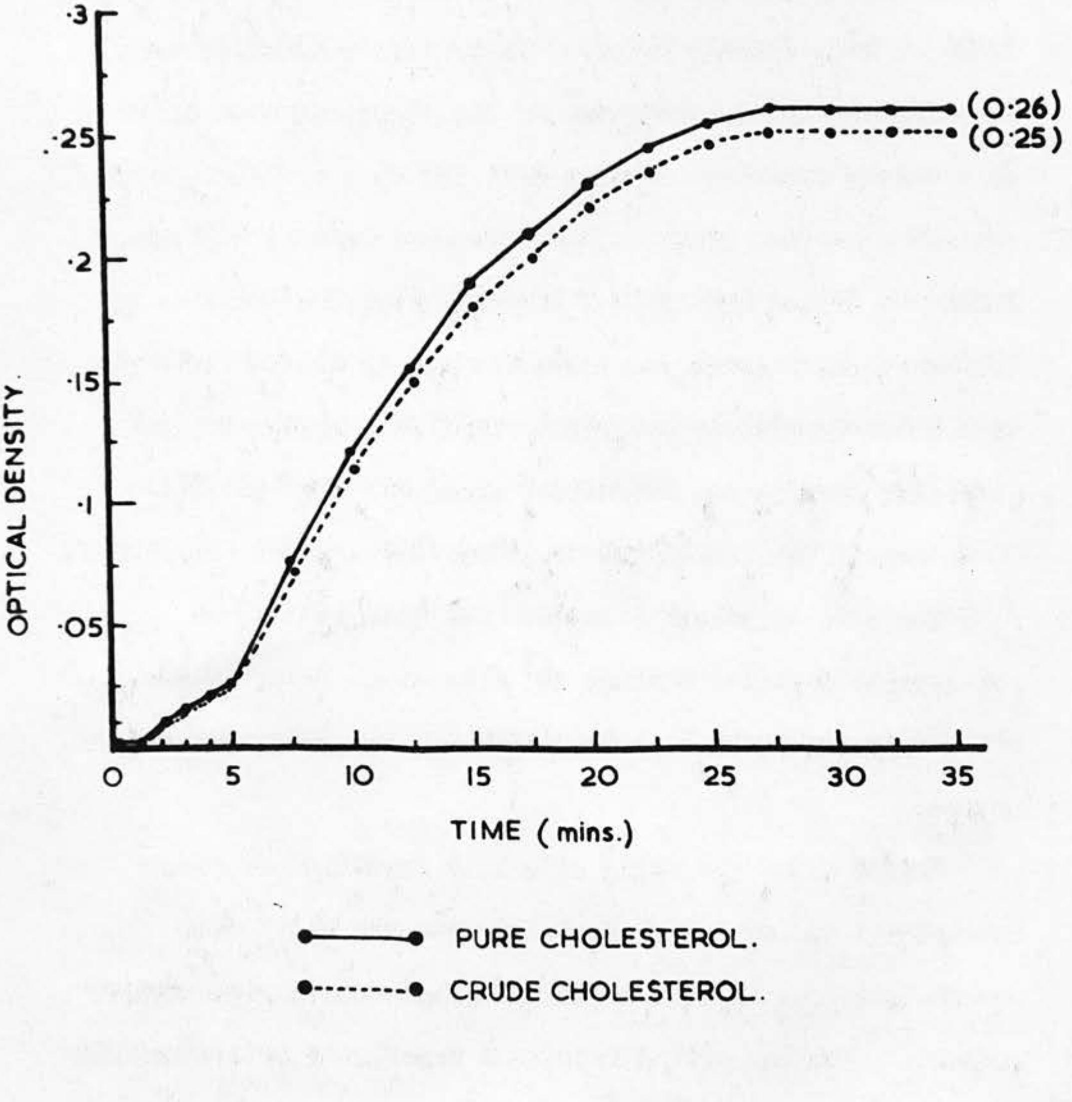
The acetate was prepared from the purified sterol by treatment with acetic anhydride and pyridine at room temperature for 48 hours. The product was recrystallised from acetone-ether yielding a white crystalline material, m.p. and mixed m.p. (with cholesterol acetate) 115 °C.

All melting points were made on a Kofler hot-stage apparatus.

(c) Colour Development with a Modified Schoenheimer-Sperry-Liebermann-Burchard Reaction.

100mg. crude cholesterol (prepared from the digitonide precipitate) were dissolved in acetone-ethanol (1:1, v/v) and the solution made up to 100ml. in a volumetric flask. Aliquots of this solution were diluted tenfold with acetone-ethanol (1:1, v/v) and 2ml. samples (containing 0.2mg. crude cholesterol) pipetted into a series of tubes. The samples were evaporated on a water-bath and then heated in an /

FIG. A.



·3
·25
·2
·15
·1
·05
OPTICAL DENSITY.
0
5
10
15
20
25
30
35
TIME (mins.)
(0·26)
(0·25)
PURE CHOLESTEROL.
CRUDE CHOLESTEROL.

an oven at 110 C for 30 minutes. The residues were dissolved by the addition of 1ml. glacial acetic acid and the tubes transferred to a water-bath maintained at 25°C ± 0.5.

The colouring reagent was made by adding 1ml. concentrated sulphuric acid to 19ml. acetic anhydride, and cooled to room temperature before use (Moore and Baumann, 1952). 2ml. freshly prepared reagent were added to each tube and the optical densities of the solutions were measured on a Unicam spectrophotometer S.P. 350 at 620 millimicrons, against a reagent blank. Readings were made at 1 minute intervals during the first 5 minutes after the addition of the colouring reagent and thereafter at 2½ minute intervals to a total time of 35 minutes. Duplicate 0.2mg. cholesterol (purified through the dibromide) standards were incubated for 30 minutes with each series of colour development experiments.

The rate of colour formation was also studied on cholesterol purified through the dibromide, using an exactly similar procedure to that described for the crude cholesterol sample.

Fig. A shows the rates of colour formation of pure cholesterol and of sterol isolated from the digitonide precipitate, on addition of the modified Schoenheimer-Sperry reagent. The two curves represent triplicate determinations and are quite comparable since the pure cholesterol standards showed good agreement.

It /

It can be seen from Fig. A that the colour development curves are of similar form and that the maximum colour given by the crude cholesterol does not include any contribution from 'fast-acting' sterols (Idler and Baumann, 1953) which might be present. The differences shown between the two curves suggest that the crude cholesterol sample includes about 4% non-chromogenic material.

APPENDIX III.

JUSTIFICATION OF THE CHLOROFORM-METHANOL EXTRACTION PROCEDURE

JUSTIFICATION OF THE CHLOROFORM-METHANOL EXTRACTION PROCEDURE

(a) Efficiency of Cholesterol Extraction from Egg Homogenates.

The contents of three 5-day eggs were pooled and homogenized with an equal weight of water in an Atomix blendor. Three 1ml. samples of homogenate were separately extracted with chloroform-methanol (1:1, v/v) and made up to 50ml. as previously described (p. 6). The protein precipitates were filtered by suction on filter paper (lipid free) and the filtrates transferred to glass-stoppered tubes. Individual samples (3ml.) of the clear filtrate were withdrawn for analysis of total cholesterol as described on p. 6.

The protein precipitates together with filter papers were re-extracted with chloroform-methanol (1:1) as before, and again made up to 50ml. Total cholesterol determinations were made on the entire filtered extracts.

A second experiment was performed in exactly the same manner on homogenates from three unincubated eggs.

The results are shown in Table A from which it can be seen that the second extraction contains as little as 1% of the amount of cholesterol present in the initial chloroform-methanol extracts.

Table A. /

Table A.

		Cholesterol (mg.)/50ml. Extract (a)	(b)	(c)
Experiment I	1st Extraction	3.20	3.20	3.20
	2nd Extraction	0.03	0.04	0.03
Experiment II	1st Extraction	3.40	3.40	3.40
	2nd Extraction	0.03	0.03	0.03

(b) Efficiency of Cholesterol and Phospholipid Extraction from Liver Homogenates.

The same procedure was also applied to homogenates of chick liver. In these experiments livers from three newly hatched chicks were pooled and homogenized in a glass-glass apparatus with 2 volumes of water. As before, filtered chloroform-methanol extracts were made up to a total volume of 50ml. 0.5ml. samples of the first extracts and 10ml. portions of the second extracts were taken for cholesterol analysis.

The results from two separate experiments are given in Table B and show that despite a high concentration of cholesterol in the homogenate, the proportion of cholesterol which could be removed from the protein precipitate by a second extraction was again of the order of 1%.

Table B. /

Table B.

		Cholesterol (mg.)/50ml. Extract		
		(a)	(b)	(c)
Experiment I	1st Extraction	21.6	21.6	21.6
	2nd Extraction	0.22	0.24	0.20
Experiment II	1st Extraction	24.8	24.8	24.8
	2nd Extraction	0.20	0.20	0.24

Samples of the above extracts were also taken (2ml., 1st extracts; 20ml., 2nd extracts) for determination of phosphorus content by the method of Allen (1940). The values thus obtained are shown in Table C from which it can be seen that less than 1% of chloroform-methanol soluble phosphorus remains in the protein precipitate after the first extraction.

Table C.

		Phosphorus (ug.)/50ml. Extract		
		(a)	(b)	(c)
Experiment I	1st Extraction	400	400	400
	2nd Extraction	25	25	23
Experiment II	1st Extraction	450	450	450
	2nd Extraction	38	25	38

(c) /

(c) Recovery of Cholesterol from Chloroform-Methanol Extracts.

An unincubated egg was homogenized with an equal volume of water. 1ml. of the homogenate was transferred to flask A containing 30ml. chloroform-methanol (1:1, v/v) and a duplicate sample pipetted into flask B which contained 25ml. chloroform-methanol and 5ml. standard cholesterol solution (1.0mg. per ml., in chloroform-methanol). The samples of homogenate were extracted as in (a) and made up to 50ml. volumes with more chloroform-methanol. Duplicate portions of each extract (3ml. from A, 1ml. from B) were removed by pipette (fitted with cotton wool filter plugs) and total cholesterol determined as before.

This procedure was repeated on homogenates from 5 unincubated eggs.

The results which are summarised in Table D show a minimum recovery of 97.4% and a maximum recovery of 100%.

Table D.

Homogenate	Cholesterol (mg.)/50ml. Extract	
	A	B
1	2.31	7.31
2	2.56	7.50
3	2.50	7.50
4	2.53	7.40
5	2.40	7.40
6	3.07	8.00

(d) /

(d) Justification of the Quantitative Determination of Phospholipid as Total Chloroform-Methanol Soluble Phosphorus.

Chloroform-methanol (1:1, v/v) extracts of liver homogenate (livers from three newly hatched chicks) were prepared in triplicate as in (b). 3ml. samples of the filtered extracts were transferred to digestion flasks, the solvent evaporated in a gentle air-stream and the residue analysed for phosphorus content by the method of Allen (1940).

A second series of 3ml. volumes were pipetted into boiling tubes and taken to dryness at a temperature of 40 °C in a stream of nitrogen. The residues were extracted four times with 5ml. portions petroleum-ether (40-60 °C), each extract being passed through a sintered-glass plate. The combined extracts from each tube were collected in digestion flasks and concentrated to dryness. Total phosphorus was determined on the residues by Allen's (1940) method. Results from two separate experiments are given in Table E from which it can be seen that 97.6 - 99.1% of the total phosphorus extracted by chloroform-methanol was soluble in petroleum-ether (40-60 °C).

Table E. /

Table E.

		Phosphorus(ug.)/50ml. Extract	
		Chloroform-methanol extract	Petroleum-ether extract
Experiment I	(1)	467	458
	(2)	467	458
	(3)	467	450
Experiment II	(1)	442	433
	(2)	442	433
	(3)	442	433

APPENDIX IV.

ATTEMPTS TO DEMONSTRATE CHOLESTEROL ESTERIFICATION IN LIVER PREPARATIONS

ATTEMPTS TO DEMONSTRATE CHOLESTEROL ESTERIFICATION IN LIVER PREPARATIONS

Homogenates prepared by treating livers from newly hatched chicks with measured volumes (1-4 times wet weight of liver) of 0.9% sodium chloride solution in a Potter--Elvehjem (1936) apparatus, were incubated for periods up to 72 hours, but in no case was any change in free and total cholesterol concentrations detected.

By analogy with the incubation of yolk-sac membrane preparations it was thought that the high concentration of ester cholesterol present in the liver homogenates might exert an inhibiting effect. A technique similar to that described for the removal of cholesterol esters from the yolk-sac was therefore applied to livers from newly hatched chicks. The resulting preparations were incubated alone and with heat inactivated human plasma (Klein, 1938).

EXPERIMENTAL AND RESULTS.

Incubation of Liver Preparations.

Livers from newly hatched chicks were pooled (6 in each pool) and homogenized in a glass-glass apparatus with twice the weight of 0.9% sodium chloride solution. The homogenates were centrifuged for 10 minutes at 3,000 r.p.m. and the yellow fatty layer (containing most of the cholesterol esters) on top of the centrifugate was discarded. Duplicate 1ml. /

1ml. samples of supernatant liquid were transferred to ground-glass stoppered tubes graduated at 10ml. and incubated for 24 hours at 37°C. Controls consisting of boiled preparations were included in each series of incubations. The saline solutions and glass-ware were sterilised by heat before use.

Acetone-ethanol (1:1) extracts were made as described on p. 20. Samples of the extracts were analysed for free and total cholesterol by the method reported on p. 6.

The results from these incubations are shown in Table E from which it can be seen that slight hydrolysis of cholesterol esters has taken place.

Table E.

Experiment No.	Percentage of Total Cholesterol in Esterified Form	
	Before Incubation	After Incubation
1	21	15
2	5	3
3	31	26
4	18	9

Incubation /

Incubation of Liver Preparations with Heat Inactivated Plasma.

Citrated human blood was centrifuged and the plasma thus obtained was heated in a sterile flask on a water bath at 60°C for 1 hour and then stored in a refrigerator at 2°C.

1ml. volumes heat inactivated plasma were pipetted into ground-glass stoppered tubes (graduated at 25ml.) followed by 0.8ml. liver preparation and 0.2ml. M acetate buffer, pH 5.7.

After incubating the tubes for 24 hours at 37°C, acetone-ethanol (1:1) extracts were made (total volume 25ml.) and free and total cholesterol determined as described in the previous experiment. All incubations were carried out in duplicate.

Controls consisting of heat inactivated plasma, boiled liver preparation and acetate buffer were included in each series of incubations.

The results which are summarised in Table F clearly show hydrolysis of cholesterol esters to have taken place. As in the previous experiment (Table E) there was no evidence of cholesterol esterification despite the presence of low concentrations of esterified cholesterol at the beginning of incubation.

Table F. /

Table F.

Experiment No.	Percentage of Total Cholesterol in Esterified Form	
	Before Incubation	After Incubation
1	22	6
2	25	14
3	31	17
4	26	13
5	24	14
6	34	25

APPENDIX V.

PURIFICATION OF REAGENTS AND SOLVENTS

PURIFICATION OF REAGENTS AND SOLVENTS

(a) Reagents.

(1) Acetic Acid: 2 l. acetic acid (B.D.H. 'Analar') were refluxed with 100g. potassium dichromate for 8 hours and then distilled. The first runnings were discarded and the main fraction boiling at constant temperature collected.

(2) Acetic Anhydride: Acetic anhydride (B.D.H. 'Analar') was distilled without pretreatment, the main constant boiling point fraction being collected.

(3) Cholesterol: Cholesterol (B.D.H., A.R. Grade) was purified through bromination. The method is essentially that of Schwenk and Werthessen (1952) and is described on page

(b) Solvents.

(1) Acetone: 2 l. acetone (commercial) were treated under reflux with 100g. potassium permanganate for 8 hours and then distilled.

(2) Chloroform: Chloroform was washed first with 5% sodium bicarbonate solution and then three times with distilled water. The chloroform was dried over sodium sulphate and distilled.

(3) Ethanol: 2 l. absolute ethanol were refluxed in the presence of 150g. potassium hydroxide for 8 hours and then /

then distilled through a fractionating column. The main middle fraction was collected.

(4) <u>Methanol</u>: Methanol was treated as for ethanol.

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I also wish to thank Dr. [illegible] of this Department for his kindness in making his paper electrophoresis apparatus available for my use.

Finally, I wish to record my indebtedness to the Agricultural Research Council, who provided a generous monetary grant to defray the expenses of these researches.

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REFERENCES

REFERENCES

Allen, R.J.L. (1940) Biochem. J. 34, 858.

Ammon, R. and Schütte, E. (1935) Biochem. Z. 275, 216.

Barrott, H.G., Fritz, J.C., Pringle, E.M., and Titus, H.W. (1938) J. Nutrit. 15, 145.

Borger, G. and Peters, T. (1933) Hoppe-Seyl. Z. 214, 91.

Boyd, G.S. (1954) Biochem. J. 58, 680.

Brody, S., Funk, E.M. and Kempster, H.L. (1932) Univ. Missouri, Coll. Agric., Agric. Exp. Stat. Res. Bull. No. 166.

Cohn, E.J. (1953) In Tullis, J.L. Blood cells and plasma proteins. (Mem. Univ. Lab. Phys. Chem., Harvard Univ., No. 2.) pp. 1-17, 337-403. New York: Academic Press.

Common, R.H., McKinley, W.P. and Maw, M.A. (1953) Science 118, 86.

Dalton, A.J. (1937) Anat. Rec. 68, 393.

Dam, H. (1928) Biochem. Z. 194, 188.

Dam, H. (1929) Biochem. Z. 215, 475.

Entenman, C., Lorenz, F.W. and Chaikoff, I.L. (1940) J. biol. Chem. 133, 231.

Gobley, /

Gobley, M. (1846) J. Pharm. Chim., Paris sér. 3, 9, 81.

Goldstein, B. and Ginzburg, M. (1937) Enzymologia 1, 369.

Heim, W.G. and Schechtman, A.M. (1954) J. biol. Chem. 209, 241.

Hevesey, G.C., Levi, H.B. and Rebbe, O.H. (1938) Biochem. J. 32, 2147.

Idler, D.R. and Baumann, C.A. (1953) J. biol. Chem. 203, 389.

Jamieson, G.S. and McKinney, R.S. (1935) Oil and Soap 12, 70.

Klein, W. (1938) Hoppe-Seyl. Z. 254, 1.

Kunkel, H.G. and Slater, R.J. (1952) J. clin. Invest. 31, 677.

Kusui, K. (1929) Hoppe-Seyl. Z. 181, 101

Le Breton, E. and Pantaleon, J. (1947) Arch. Sci. physiol. 1, 199.

Lecanu (1829) Cited by Gobley, M. (1846) J. Pharm. Chim., Paris sér. 3, 9, 81.

Malmros, H. and Swahn, B. (1953) Acta med. Scand. 145, 361.

Man, E.B. and Gildea, E.F. (1932) J. biol. Chem. 99, 43.

McKinley, W.P., Oliver, W.F., Maw, W.A. and Common, R.H. (1953) Proc. Soc. exp. Biol., N.Y. 84, 346.

Menozzi, /

Menozzi, A. (1908) R.C. Accad. Lincei, sér. 5, 17:1sim., 91-94. Cited by Bills, C.E. (1935) Physiol. Rev. 15, 1.

Moore, P.R. and Baumann, C.A. (1952) J. biol. Chem. 195, 615.

Mueller, J.H. (1915) J. biol. Chem. 21, 23.

Nikkila, E. (1953) Scand. J. clin. Lab. Invest. 5, suppl. 8.

Potter, V.R. and Elvehjem, C. (1936) J. biol. Chem. 114, 495.

Roffo, A.H. and Azaretti, I. (1926) Bol. Inst. Med. exp. Cáncer B. Aires 2, 629.

Schjeide, O.A. (1954) J. biol. Chem. 211, 355.

Schoenheimer, R. and Dam, H. (1932) Hoppe-Seyl. Z. 211, 241.

Schoenheimer, R. and Sperry, W.M. (1934) J. biol. Chem. 106, 745.

Serono, C., Montezemolo, R. and Balboni, G. (1936) Rass. Clin. Terap. Anno 35, Fasc. 5.

Skarzynski, B. (1936) Bull. int. Acad. Cracovie, Cl. Méd. 437.

Sperry, W.M. and Webb, M. (1950) J. biol. Chem. 187, 97.

Stamler, J., Bolene, C., Harris, R. and Katz, L.N. (1950) Circulation 2, 714.

Stamler, J., Bolene, C., Levinson, E. and Dudley, M. (1950) Endocrinology 46, 382.

Stewart, C.P. and Hendry, E.B. (1935) Biochem. J. 29, 1677.

Stoddard, J.L. and Drury, P.E. (1929) J. biol. Chem. 84, 741.

Stokes, /

Stokes, W.M., Fish, W.A. and Hickey, F.C. (1953) J. biol. Chem. 200, 683.

Sutherland, T.M. (1953) Ph.D. Thesis, Edinburgh.

Thannhauser, S.J. and Schaber, H. (1923) Hoppe-Seyl. Z. 127, 278.

Windaus, A. (1910) Hoppe-Seyl. Z. 65, 110.

Windaus, A. and Stange, O. (1936) Hoppe-Seyl. Z. 244, 218.

Zorn, C.M. and Dalton, A.J. (1936) Proc. Soc. exp. Biol., N.Y. 35, 451.