THE EFFECTS OF DIETHYLSTILBESTROL AND CHOLESTYRAMINE ON LIPID SYNTHESIS IN THE CHICK AND JAPANESE QUAIL

by 6408

ANNETTE C. REYNOLDS

B. A., West Chester State College, 1967

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

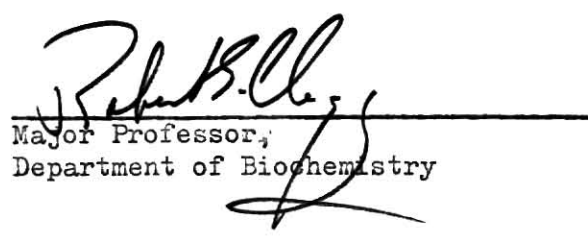
MASTER OF SCIENCE

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

Approved by:



Major Professor,
Department of Biochemistry

TABLE OF CONTENTS

ILLEGIBLE DOCUMENT

THE FOLLOWING DOCUMENT(S) IS OF POOR LEGIBILITY IN THE ORIGINAL

THIS IS THE BEST COPY AVAILABLE

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

It has been known for many years that the liver contributes a great deal to the circulating pool of cholesterol. More recently it was shown that other organs, in particular the small intestine, also made substantial contributions to this circulating cholesterol.

Diethylstilbestrol, an artificial estrogen, has been used in this and other laboratories to induce the production of phosphorus-containing components in the blood of cockerels. Another effect of diethylstilbestrol treatment was a large increase of serum lipid levels. It was found that certain drugs, including cholestyramine, lowered the elevated levels of serum cholesterol in estrogen-treated birds.

Because of the previous work on the effects of these two drugs, diethylstilbestrol (DESB) and cholestyramine, on serum cholesterol levels in birds, an exploration of their effects upon the synthesizing activity of several tissues was initiated. Diethylstilbestrol and cholestyramine appear to have different modes of action; DESB apparently acts systemically whereas the cholestyramine acts in the gut. The tissues chosen were the liver and the intestine because of their high cholesterol synthesizing activities and because the cholestyramine apparently acts in the gut and not the liver.

At first it was expected that the diethylstilbestrol would raise synthesis levels in the liver and intestine and that cholestyramine would have a depressing effect, at least on the intestinal samples. However, a closer look at the pertinent literature indicated that the cholestyramine treatment could lead to increased synthetic activity in both organs and that estrogens could conceivably have little or no effect upon cholesterol synthesis. As a result of these seemingly conflicting reports it was decided

to explore the effects of DESB and cholestyramine upon the cholesterol synthesizing activities of liver and intestinal slices and to compare the results obtained with previous work from this laboratory upon serum cholesterol levels.

LITERATURE REVIEW

For many years the liver was considered to be the greatest contributor to cholesterol circulating in the body (1-4). Any changes in the serum cholesterol levels were assumed to have resulted from changes in hepatic biosynthesis of cholesterol. Other organs have been known to produce cholesterol but it was not believed they contributed significant amounts to the circulating pool (5). Recently Lindsey and Wilson (6), examined the contribution of other organs to the circulating pool of cholesterol and the intestine appears to be the most active organ, other than the liver. Dietschy and Siperstein (7) further studied cholesterol synthesis by the gastrointestinal tract, and found that, although synthesis occurs all along the gastrointestinal tract in rats, rates of synthesis found in the stomach, ileum and distal colon were greater than in other portions. In addition, sterol synthetic activity was found almost exclusively in the layer of the small intestine containing the intestinal crypts as contrasted with the intestinal villi and smooth muscle which showed no synthetic activity.

It has been demonstrated that either the addition of cholesterol to the diets of rats or fasting inhibits hepatic synthesis by affecting the conversion of β-hydroxy-β-methyl glutaryl coenzyme A to mevalonic acid (2,8-14). Dietschy and Siperstein (15) reported that cholesterol feeding or fasting does not inhibit intestinal synthesis of cholesterol, but other investigators (16-18) noted a depression of intestinal synthetic activity after prolonged fasting.

Estrogens have been shown to affect cholesterol levels. Levin (19) reported that diethylstilbestrol caused a loss of cholesterol from the adrenal glands and from the serum of rats. The reduced levels were main-

tained for 34 days with little indication of the condition reversing itself. Rosenman et al. (20) reported that estrogens have a depressing effect upon hepatic cholesterol synthesis. More recently, Mukherjee and Bhose (21) demonstrated 17β-estradiol inhibition of the hydroxymethyl glutaryl-Co A condensing enzyme in rat liver microsomes.

In the bird, estrogens have been shown to raise the levels of blood lipids in both male and female animals (22). DESB has been used to produce atheromatosis in the aortas of birds (23).

Bile acids have been shown to control cholesterol biosynthesis and transport in the rat. Figure 1, which was adapted from the work of J. M. Dietschy (24) illustrates the interrelationship between hepatic and intestinal cholesterol metabolism and the role of bile acids. Cholesterol esters in the lumen are hydrolyzed to cholesterol by pancreatic cholesterol esterase (25). This reaction requires bile acids. Solubilization of cholesterol with other lipid components also requires bile acids to facilitate diffusion across the mucous membrane of the intestinal epithelium (25,26). Cholesterol in the intestinal wall is also derived from de novo synthesis from acetate. This intestinal synthesis of cholesterol is inhibited by the presence of bile (27). The intestinal pool of cholesterol is incorporated into lipoproteins, especially chylomicrons, and then enters the general body circulation via the enterolymphatic circulation. The passage of the lipoproteins into the lymph requires the presence of bile acid (28). Cholesterol synthesized in the liver also contributes to the circulating pool of sterols.

Bile acids are secreted from the liver into the intestinal lumen via the common bile duct. After absorption across the gut, the acids are returned via portal circulation to the liver.

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

THIS IS AS RECEIVED FROM CUSTOMER.

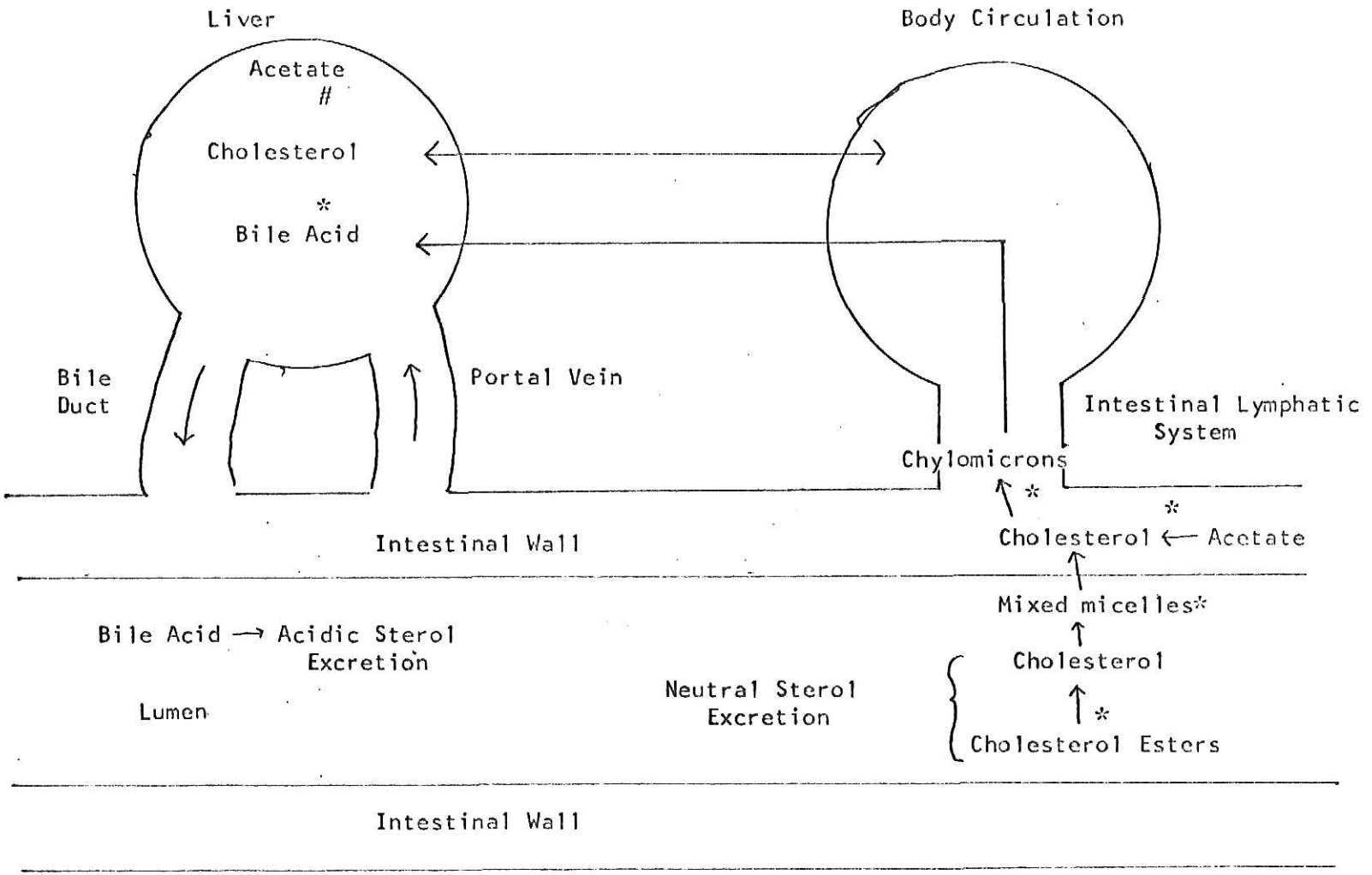


Figure 1. The interrelationship between hepatic and intestinal cholesterol metabolism and the role of bile acids. *indicates control mediated by bile acids. #indicates control mediated by cholesterol.

It had been thought that bile acids exert a negative control on hepatic cholesterologenesis from acetate and the synthesis of bile acids from cholesterol. Seitz and von Brand (29) and Fimognari and Rodwell (30) have reported that the addition of bile acid in vitro to subcellular preparations or to liver slices inhibits synthesis of cholesterol. Biliary diversion (31,32) or cholestyramine feeding (33) increases hepatic cholesterologenesis. These results would seem to indicate that bile acids exert a direct effect upon hepatic sterol synthesis.

It has been shown, however, that bile acids do not directly influence cholesterol synthesis from acetate in the liver. A rise is seen in hepatic cholesterologenesis upon ligation of the common bile duct (34) as well as when an animal has been subjected to cholestyramine feeding or biliary diversion. Weis and Dietschy (35) showed that both biliary diversion and biliary obstruction increased sterol synthesis in the liver. In the case of biliary obstruction the bile acid level of the liver is elevated. Restoration of the enterohepatic circulation of bile acid with biliary diversion fails to prevent the subsequent rise in synthetic activity.

Weis and Dietschy further suggest that the changes noted in hepatic synthetic activity result from changes in the enterolymphatic circulation of cholesterol. They found that intestinal lymphatic diversion causes the same increase in synthetic activity as biliary diversion. In addition they prevented the increase in the rate of hepatic sterol synthesis in rats with biliary diversion by infusing cholesterol in the form of chylomicrons (35).

The results of these studies was summarized by Dietschy (24). In an animal where there is an absence of bile acid in the intestine due to interruption of the enterohepatic circulation, there is malabsorption of cholesterol from the lumen and decreased passage of cholesterol into the intes-

tinal lymph. Therefore the rates of cholesterol synthesis in both the liver and intestine are greatly increased because of the loss of inhibition in both organs; i.e., bile acids are not inhibiting intestinal synthesis and the cholesterol synthesized in the gut is not reaching the liver. In the case of intestinal lymphatic diversion, hepatic cholesterologenesis is increased because of the loss of feedback inhibition since cholesterol from the gut does not reach the liver. However, the rate of intestinal synthesis does not rise because the enterohepatic circulation of bile is intact.

Cholestyramine is a quaternary ammonium anion exchange resin in which the basic groups are attached to a styrene-divinyl benzene copolymer skeleton. It lowers serum cholesterol levels in several species of animals when taken orally. Cholestyramine is used in humans (under the trade name Questran from Mead Johnson) to lower plasma cholesterol levels (36). This drug is also effective in lowering blood cholesterol levels in the dog (37, 38). In the chick, Tennent et al. (37) reduced hypercholesterolemia which was induced by a diet containing cholesterol and cottonseed oil. Results of studies in this laboratory show reduction of serum cholesterol levels in cockerels treated with diethylstilbestrol when fed cholestyramine as 1% of the feed (39). However, cholestyramine had no effect on plasma cholesterol levels in swine or rats (33,40).

Cholestyramine is not absorbed by the body, therefore its action must take place in the gut (41). This drug has been shown to greatly increase fecal bile acid excretion (33,42). As shown by Dietschy (24), this results in an interruption of enterohepatic circulation, and hence, greatly increases the rate of cholesterologenesis in the liver and gut.

METHODS AND MATERIALS

Treatment of Animals. White Rock cockerels of eight weeks of age were divided into four groups: controls; birds treated with 1.25 mg of diethylstilbestrol (DESB) for seven days; birds treated with cholestyramine as 1% of their feed for seven days; and birds treated with both the DESB and cholestyramine. The DESB was dissolved in propylene glycol at a concentration of 2.5 mg/ml. All animals were fed regular laboratory feed and water was given ad libitum.

Tissue Isolation. The birds were sacrificed by severing the jugular vein. The liver and intestine were excised immediately. Only the portion of the small intestine between the duodenal fold and the ceca was used. The tissues were washed with cold physiological saline and placed in oxygenated Krebs bicarbonate buffer, pH 7.4 at 0° C. The Krebs bicarbonate buffer had the following composition:

.90% NaCl (.154 M)	100 parts
1.15% KCl (.154 M)	4 parts
1.22% $CaCl_2$ (.11 M)	3 parts
2.11% KH_2PO_4 (.154 M)	1 part
3.82% $MgSO_4 \cdot 7H_2O$ (.154 M)	1 part
1.30% $NaHCO_3$ (.154 M) - gassed with CO_2 for one hour	21 parts

The complete buffer was gassed for 10 minutes with 5% CO_2, 95% O_2. All solutions were prepared five times the given concentrations and diluted as needed. All solutions were stored separately in a cold room or refrigerator.

Tissue Preparation and Incubation.

Tissue Homogenates. Intestinal tissue was cut into ¼ to ½ inch lengths

and placed in a micro Waring blendor with enough oxygenated Krebs bicarbonate buffer such that there were 2 ml buffer per gram of tissue (wet weight). This system was homogenized for 30 seconds and the volume measured. Homogenate corresponding to approximately 0.5 g tissue was used in each incubation flask with 8 ml oxygenated buffer and 50 micromoles of sodium acetate.

Tissue slices. Liver slices were prepared using a Stadie Riggs tissue slicer. Intestinal slices were prepared manually. Approximately 500 mg of tissue (wet weight) were placed into incubation flasks containing 5 ml oxygenated Krebs bicarbonate buffer and five micromoles sodium acetate in .1 ml of solution. In experiments where radioactive tracers were used, the 5 micromoles sodium acetate contained 1 microcurie sodium acetate-2 C14 (ICN Chemical, Irvine, California). The flasks were incubated at 37° C for 90 minutes in a shaking water bath. The reactions were stopped by addition of 1 ml saturated potassium hydroxide solution. The samples were saponified at 150° C and 15 psi for 30 minutes in an autoclave. After cooling , an equal volume of ethanol was added and the samples extracted with three-five milliliter portions of pentane.

Isolation of Steroids. Three different procedures for quantitatively isolating cholesterol and related steroids were attempted.

1. Gravimetric analysis of sterol digitonides (43). The non-saponifiable lipids were dissolved in 4 ml of acetone-ethanol (1:1 v/v), placed in tared tubes and 1 ml each of water and digitonin solution (2% in 80% ethanol, w/v) added. The tubes were left standing overnight. The tubes were centrifuged and the supernatant discarded. The precipitate was washed with three 1.5 ml portions of 80% ethanol and three 1.5 ml portions of diethyl ether. The ether was evaporated by passing a stream of nitrogen over the tubes. The tubes were placed in an oven at 110° C for 1 hour, in

vacuo overnight and then weighed.

2. Gas chromatographic analysis of nonsaponifiable lipids (44). A Barber-Colman model 5000 gas chromatograph was used. A 2 meter long U-shaped column containing 3% QF-1 on 100-120 mesh Gas-Chrom Q was obtained from Applied Science Laboratories, State College, Pa. The following conditions were employed: injection temperature, 270° C; column temperature, 230° C; detector temperature, 240° C; gas flow, 90 ml/min. The gas chromatograph was equipped with a hydrogen flame ionization detector. Nitrogen was used as the carrier gas.

3. Thin layer chromatography and liquid scintillation counting. After evaporation of the pentane solvent, the steroids were redissolved in 1 ml of chloroform. A 100 microliter aliquot was spotted on a silica gel thin layer chromatography plate (supplied by Applied Science Laboratories, State College, Pa.) and developed in benzene-ethyl acetate, 5:1 (v/v). The steroids were visualized by spraying with .005% Rhodamine G solution (w/v) and viewing under ultraviolet light. The thin layer plates were sprayed with Neatan plastic spray (Brinkman Chemical), and allowed to dry, and the spots scraped into scintillation vials.

The scintillation cocktail consisted of 0.015% p-bis 2-(5-phenyloxazolyl)-benzene and .30% 2,5-diphenyloxazole in toluene. All samples were counted in a Beckman liquid scintillation counter, model LS 200B. Results were calculated as disintegrations per minute (DPM) per 100 mg tissue (wet weight).

RESULTS AND DISCUSSION

An attempt was made to employ a gravimetric analysis of sterol digitonides but it was not eventually used for several reasons. The first of these is the fact that digitonin is specific for sterols with an -OH group in position number 3. While cholesterol is the major sterol found in the tissues, there was the possibility that related compounds would contribute significantly to the weight of the sample since this assay deals with microgram quantities. Another reason for not using this method of analysis was the fact that it was difficult to obtain reproducible results primarily because of the small amounts of sterols present.

An attempt was made to analyze the nonsaponifiable lipids using the gas chromatograph. The results of the gas chromatographic work is summarized in Tables 1 and 2.

Originally tissue homogenates were used with the gas chromatographic system. This method was abandoned because it was difficult to obtain uniform and accurate amounts of homogenate per incubation flask. In addition, because of the inconclusive results of the gas chromatographic work (see Table 1), and the fact that the synthesizing capabilities of this homogenate system were not demonstrable, it was decided to use tissue slices. The intestinal slices of each bird were subjected to one of three treatments: saponification before incubation (S), incubation at 37° C for 2 hours without additional acetate added to the incubation mixture (NA), and incubation at 37° C for 2 hours with 50 micromoles of sodium acetate added (A). The rationale behind this treatment was that the saponified sample would give the cholesterol levels in the intact animal, the sample incubated with no additional acetate would give information about the acetate pool found in

Table 1. Milligrams cholesterol per gram of chicken intestine as determined by gas chromatography.

Animal Treatment		Incubation Treatment*	Mg cholesterol per g tissue (median value for 9 samples ± standard deviation)
None	1	S	.363 ± .163
		NA	.464 ± .179
		A	.780 ± .194
	2	S	1.813 ± .309
		NA	2.193 ± .570
		A	1.552 ± .319
	3	S	1.351 ± .327
		NA	1.167 ± .569
		A	1.525 ± .579
DESB	1	S	2.166 ± .294
		NA	1.953 ± .222
		A	1.945 ± .302
	2	S	1.586 ± .171
		NA	1.647 ± .389
		A	1.264 ± .226
	3	S	1.877 ± .289
		NA	1.694 ± .282
		A	1.756 ± .282
Cholestyramine plus DESB	1	S	1.726 ± .483
		NA	1.496 ± .320
		A	1.849 ± .386
	2	S	2.488 ± .309
		NA	2.720 ± .388
		A	2.138 ± .379
	3	S	2.358 ± .525
		NA	2.229 ± .476
		A	1.982 ± .427

*S=saponified before incubation; NA=incubated without additional acetate; A=incubated with 50 micromoles additional sodium acetate.

Table 2. Milligrams cholesterol per gram of quail intestine as determined by gas chromatography.

Animal Treatment	Animal Number	Incubation Treatment*	Mg cholesterol per g tissue (median value for 3 samples ± standard deviation)
None	1	S	1.232 ± .0587
		NA	1.282 ± .173
		A	1.208 ± .117
	2	S	1.216 ± .0397
		NA	1.268 ± .0769
		A	1.214 ± .291
	3	S	1.300 ± .157
		NA	1.378 ± .0120
		A	1.279 ± .0797
Cholestyramine	1	S	1.852 ± .484
		NA	1.546 ± .139
		A	1.476 ± .0744
	2	S	1.804 ± .129
		NA	1.643 ± .116
		A	1.514 ± .432
	3	S	1.655 ± 1.285
		NA	1.380 ± .102
		A	1.514 ± .271
Diethylstilbestrol	1	S	1.244 ± .092
		NA	1.315 ± .186
		A	1.110 ± .0233
	2	S	1.270 ± .0165
		NA	1.160 ± .190
		A	1.125 ± .182
	3	S	1.452 ± .244
		NA	1.292 ± .309
		A	1.162 ± .136
Diethylstilbestrol-Cholestyramine	1	S	1.841 ± .105
		NA	1.456 ± .0453
		A	1.366 ± .0721
	2	S	1.477 ± .183
		NA	1.540 ± .163
		A	1.676 ± .0917
	3	S	1.310 ± .189
		NA	1.354 ± .199
		A	1.038 ± .201

the gut and that the sample incubated with additional acetate would give information concerning de novo synthesis of cholesterol.

This gas chromatographic system of analysis was used with 2 sets of birds. The first set was a group of chickens consisting of control birds, diethylstilbestrol-treated birds, and cholestyramine-DESB treated birds. The second set of birds were quail subjected to the same treatments as above and cholestyramine feeding. The quail were employed because of the unavailability of chickens at this time.

It can be seen from the data in Tables 1 and 2, that a great deal of variation in the amounts of cholesterol was exhibited in each incubation system. There appeared to be no pattern to the cholesterol values obtained. There are several possible explanations for these results: 1) no synthesis of cholesterol occurred in the incubated flasks, i.e., the system did not work; 2) 50 micromoles of sodium acetate were not a sufficient amount of additional substrate to drive the reaction; 3) the amount of newly synthesized cholesterol was so small that it could not be detected by this method; or 4) de novo synthesis of cholesterol occurred, but since the reaction is reversible, the newly formed cholesterol was subsequently broken down resulting in no net synthesis of this sterol.

Because the explanation could not be ascertained from any of our data this method of cholesterol analysis was abandoned in favor of combining thin layer chromatography and radiotracers. According to the results reported by Dietschy and Siperstein (15) we expected to find greater than 90% of the incorporated label in the cholesterol spot. These authors assumed that the incorporation of the labeled acetate could be equated with net synthesis of cholesterol.

Figures 2 and 3 illustrate typical thin layer plates from the two sets of

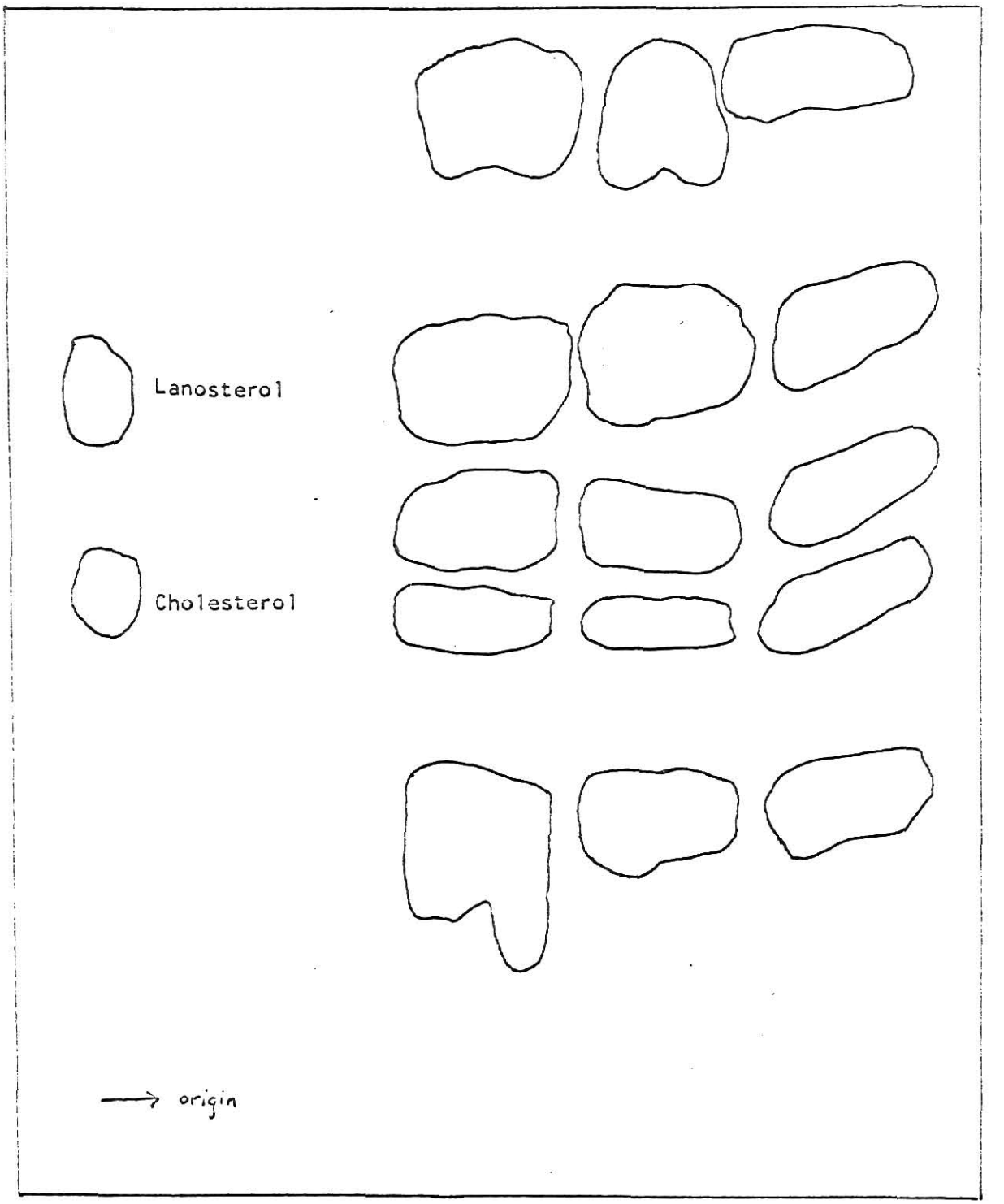


Figure 2. Typical thin layer chromatogram from Group A birds.

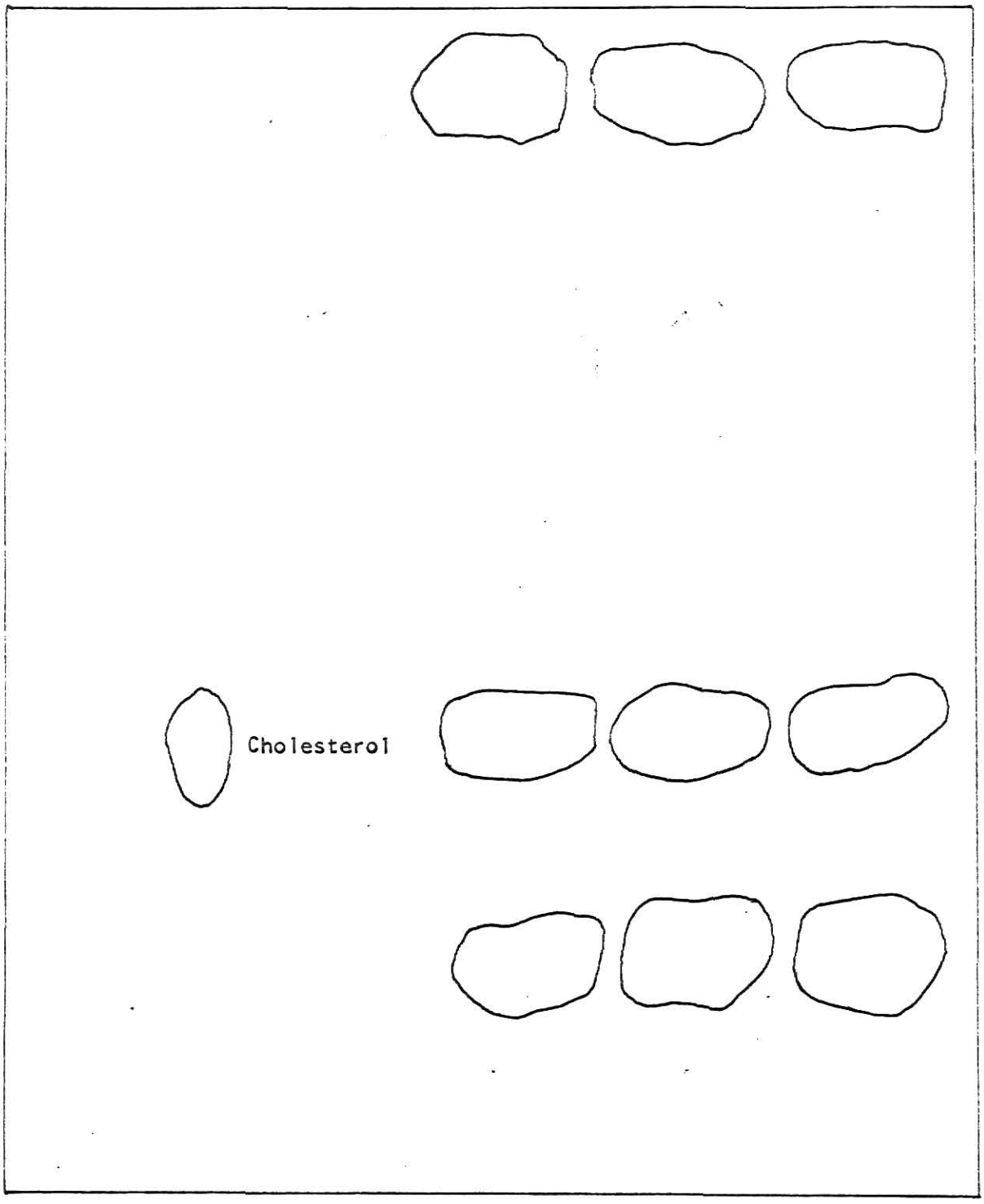


Figure 3. Typical thin layer chromatogram from Group B birds.

birds used for the radiotracer work. The spots found were tentatively identified from the work of Dietschy and Siperstein (15). It was not possible to obtain reference standards of the methostenols (4α-methyl- Δ^7-cholestenol and 4α-methyl- Δ^8-cholestenol), and Δ^7cholestenol. Because the sterols were not first precipitated with digitonin and because cholestane appeared in the gas chromatographic work, this steroid was also run in the thin layer chromatography system used. It was found that cholestane traveled with the solvent front. Because of the questionable homogeneity of the compound found traveling with the solvent front, it has been labeled cholestane with the understanding that it could, conceivably, contain other compounds.

The results of the work using sodium acetate-2C14 are summarized in Tables 3, 4, 5, and 6 and Figures 4 through 9. The first set of data (Group A) corresponds to results obtained by incubating intestinal tissue of chickens with sodium acetate-2C14 as described in the Methods section. As can be seen from the data in Table 3 and Figures 4, 5, and 6, there was much scatter among the values obtained, and it was felt that few valid conclusions could be drawn. In most cases, the acetate incorporated into the cholesterol fraction represented less than 70% of the radioactivity found in the pentane extract.

A study was undertaken to determine how rapidly the reactions that led to the synthesis of cholesterol reversed themselves. In this experiment two flasks were used. One contained only buffer and cholesterol-4C14. The other flask contained buffer, labeled cholesterol and intestinal tissue slices. The same amount of cholesterol was added to each flask. The flasks were then incubated, saponified and extracted with pentane as described previously. A small amount of unlabeled cholesterol was added to the "blank" extract to facilitate visualization on the thin layer plate. The pentane

Table 3. Disintegrations per minute per gram of intestinal tissue for steroids separated by thin layer chromatography from a pentane extract (Group A). Mean value of 5 samples ± standard deviation.

Animal Treatment	Animal Number	Δ^7-cholestenol	cholesterol-cholestanol	4α-CH_3-Δ^7 (and Δ^8) cholestenol	lanosterol	cholestane (solvent front)
None	1	1589 ± 511	3688 ± 2191	1665 ± 343	809 ± 306	1127 ± 211
	2	1052 ± 294	4387 ± 233	853 ± 331	1152 ± 815	779 ± 254
	3	434 ± 13	4600 ± 3636	1532 ±1531	506 ± 458	968 ± 893
Cholestyramine	1	1734 ± 607	13605 ± 257	1579 ± 257	2072 ± 1025	1082 ± 360
	2	2013 ±1279	4220 ± 2188	1009 ± 277	623 ± 516	864 ± 382
	3	1687 ± 549	4923 ± 1662	1601 ± 460	999 ± 332	737 ± 329
Diethylstilbestrol	1	1066 ± 259	3748 ± 1575	1474 ± 127	551 ± 455	869 ± 208
	2	1409 ± 725	6936 ± 2963	1111 ± 224	1033 ± 966	1227 ± 390
	3	1013 ± 239	5365 ± 2262	1202 ± 97	2581 ± 202	904 ± 456
Diethylstilbestrol-Cholestyramine	1	2444 ± 597	2624 ± 725		1151 ± 655	992 ± 230
	2	1746 ± 803	2669 ± 1124	936 ± 350		960 ± 270
	3	2429 ± 801	2537 ± 520	1178	311 ± 267	544 ± 194

Table 4. Disintegrations per minute per .1 ml aliquot of pentane extract of buffer and tissue incubation mixture containing ^{14}C-cholesterol.

Blank		19,274.6			
Tissue incubation	744.6	14,063.8	139.7	22.3	136.5

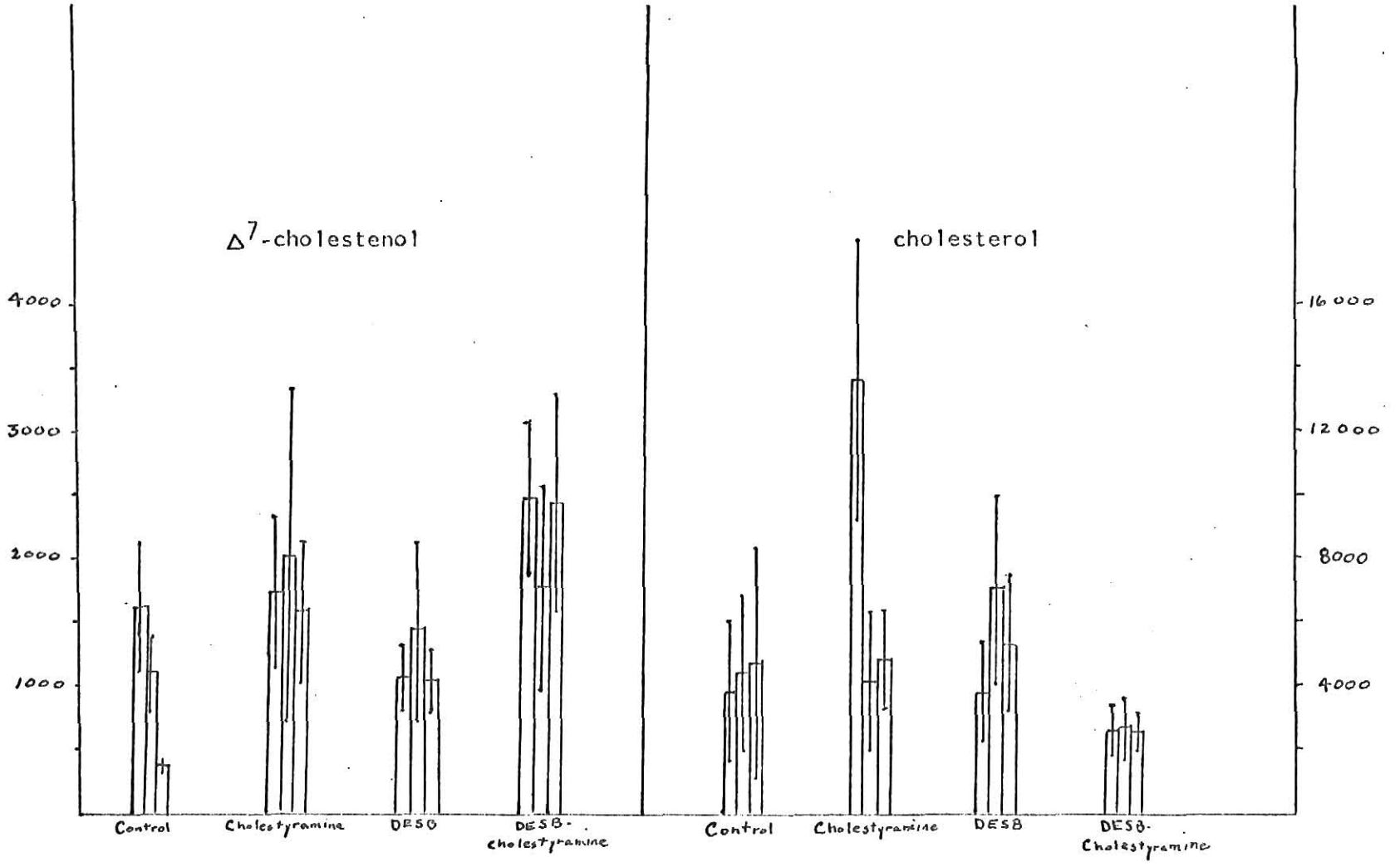


Figure 4. Disintegrations per minute per gram intestinal tissue of Δ^7-cholestenol and cholesterol.

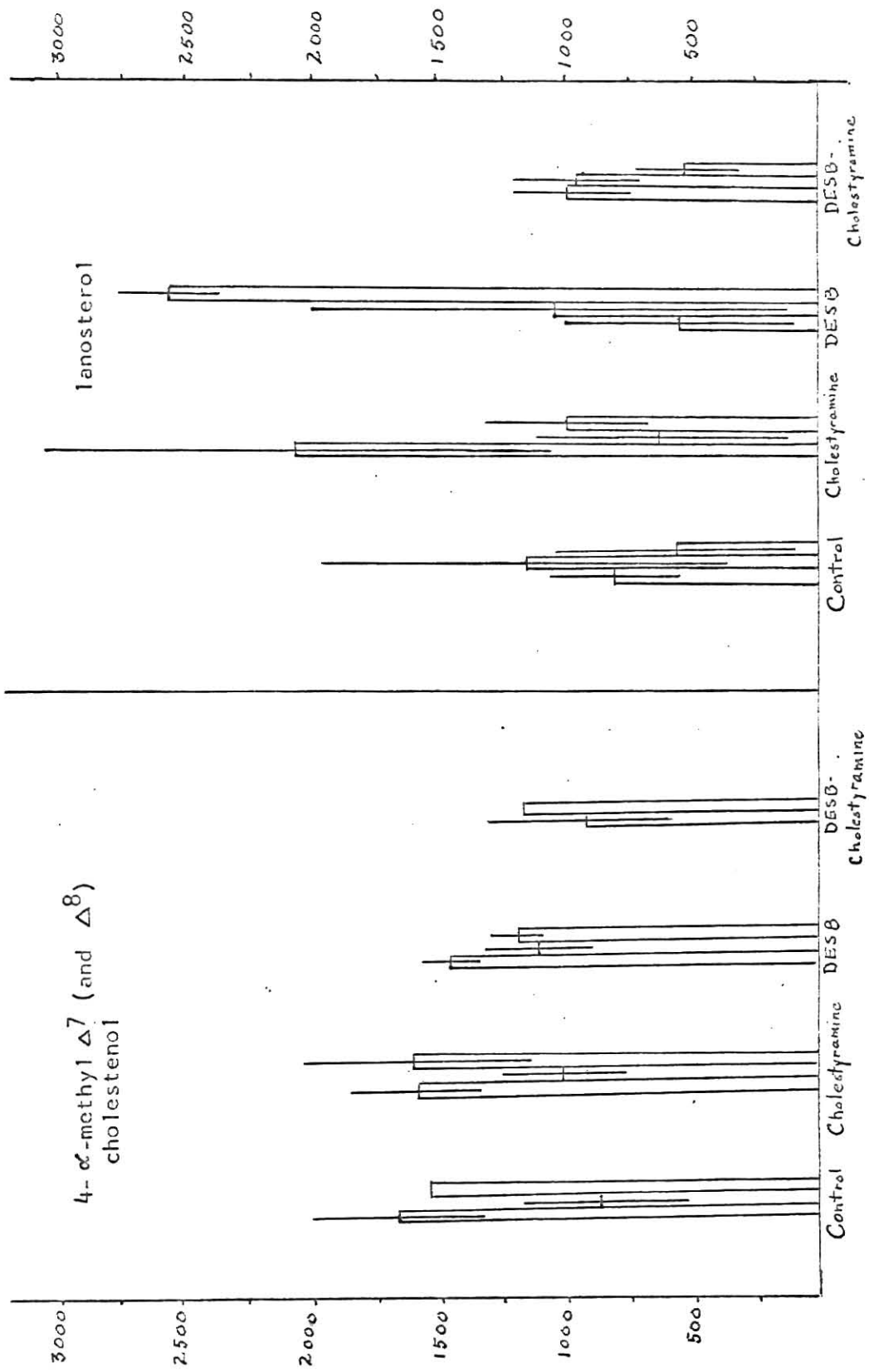


Figure 5. Disintegrations per minute per gram intestinal tissue of 4- α-methyl Δ^7 (and Δ^8) cholestenol and lanosterol.

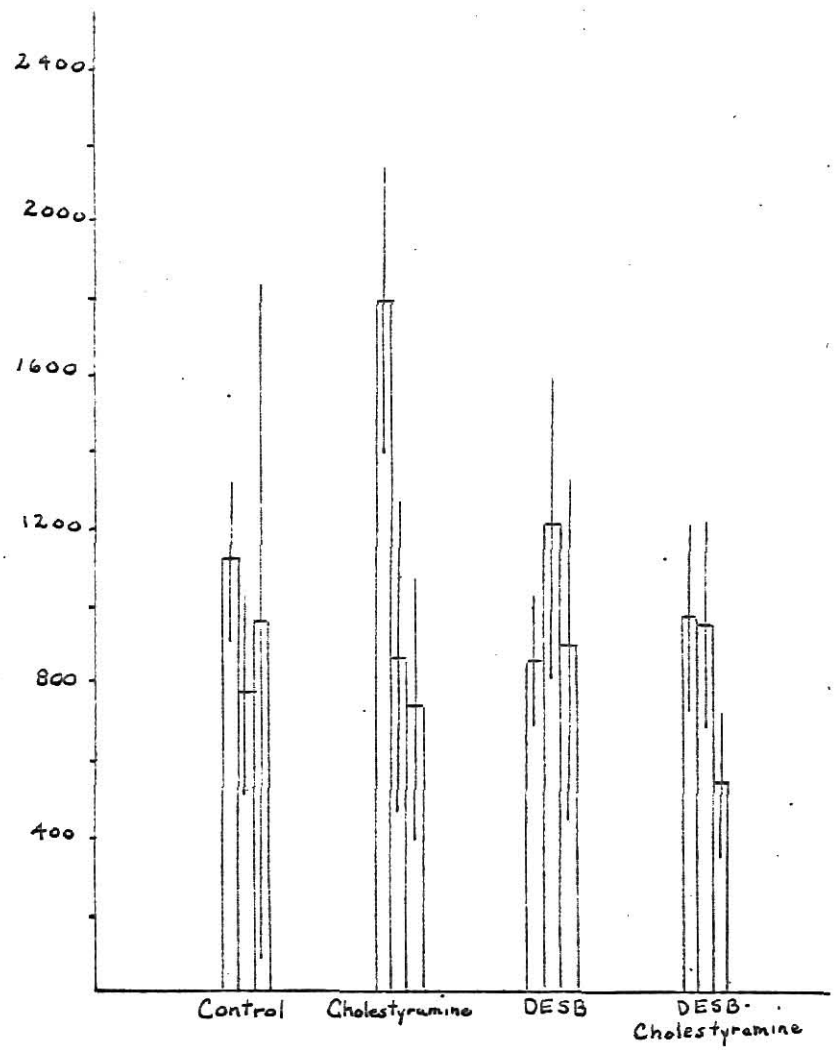


Figure 6. Disintegrations per minute per gram intestinal tissue of cholestane (solvent front).

extracts were subjected to thin layer chromatography and the radioactivity in each spot determined. The data obtained are summarized in Table 4. This experiment indicated that, indeed, the reactions were reversible and that approximately 27% of the labeled cholesterol that was present in the initial incubation mixture could not be recovered as cholesterol. Radioactivity was found in the other sterol fractions. Since all of the radioactivity added to the initial system could not be recovered in the steroid fraction, i.e., the pentane extract which was spotted on the thin layer plate, it was assumed that some of the labeled cholesterol was converted to cholesterol precursors occurring before lanosterol. The possibility also exists that the cholesterol was further metabolized. This possibility was not investigated in this study.

Because of the inconclusive results from the first group of chickens used, it was decided to rerun the radiotracer experiment using not only intestinal tissue, but liver tissue as well (Group B). These data are summarized in Tables 5 and 6 and Figures 7, 8, and 9. The results this time showed much less scatter than previously.

Although essentially the same system was used with both Group A and Group B birds, somewhat different results were obtained. In Group A, most samples gave 5 spots in the thin layer chromatography. The amount of radioactivity incorporated in the cholesterol fraction was generally less than 70% of the total amount in the steroid fraction. In most of the samples of Group B only three spots were found in the thin layer chromatography and the amount of radioactivity found in the cholesterol spot approached the 80-90% figure obtained by Dietschy and Siperstein.

There were slight differences in the treatments of Groups A and B. The group B birds were 1-4 weeks younger that the Group A birds. No more

Table 5. Disintegrations per minute per gram of intestinal tissue for steroids separated by thin layer chromatography from a pentane extract (Group B). Median value for 3 samples ± standard deviation.

Treatment	Animal Number	Δ^7-cholestenol	cholesterol-cholestanol	cholestane (solvent front)
None	1	309.0 ± 204.4	1037.3 ± 424.3	410.6 ± 13.2
	2	173.6 ± 24.8	2741.5 ±2038.7	421.7 ± 110.3
	3	143.7 ± 30.1	2505.7 ± 753.8	439.6 ± 101.6
Cholestyramine	1	712.0 ± 372.3	5521.7 ± 770.8	1325.3 ± 301.4
	2	197.7 ± 62.5	4092.8 ± 1050.4	1396.7 ± 557.3
	3	557.9 ± 37.9	11017.0 ± 1814.4	1611.0 ± 11.5
Diethylstilbestrol	1	379.2 ± 187.1	1545.8 ± 660.9	514.1 ± 109.9
	2	176.5 ± 38.3	1324.2 ± 536.9	425.6 ± 170.5
	3	271.4 ± 166.8	2185.0 ± 624.3	555.4 ± 223.8
Diethylstilbestrol-Cholestyramine	1	292.0 ± 168.1	2975.7 ± 214.2	613.5 ± 89.8
	2	316.0 ± 101.6	2144.6 ± 228.9	725.8
	3	271.6 ± 128.1	2517.7 ±1866.6	588.4 ± 282.1

Table 6. Disintegrations per minute per gram of liver tissue for steroids separated by thin layer chromatography from a pentane extract (Group B). Median value for 3 samples ± standard deviation.

Treatment	Animal Number	Δ^7-cholestenol	cholesterol-cholestanol	cholestane (solvent front)
None	1	1668.1 ± 585.2	29926.9 ± 8652.3	2427.0 ± 753.0
	2	1261.5 ± 256.0	14562.3 ± 1040.5	2168.0 ± 181.7
	3	698.8 ± 103.5	37490.0 ± 2608.6	1065.1 ± 147.9
Cholestyramine	1	1514.0 ±1672.2	40433.0 ±18251.3	4688.2 ±2540.7
	2	614.5 ± 180.7	60657.1 ± 3168.7	2504.4 ± 471.5
	3	1968.7 ± 283.0	74387.2 ± 5577.5	4924.3 ± 205.8
Diethylstilbestrol	1	1276.2 ± 357.0	43919.0 ± 804.4	7439.3 ±1096.2
	2	2088.7 ± 725.2	24424.4 ± 643.6	3116.8 ±1308.8
	3	911.8 ± 157.1	13530.6 ± 3511.1	108.1 ± 87.2
Diethylstilbestrol-Cholestyramine	1	1656.0 ± 431.6	50089.5 ± 1375.0	8916.7 ±1014.0
	2	1671.0 ± 403.5	51039.3 ± 8128.3	4309.1 ± 465.7
	3	545.6 ± 271.5	14675.6 ± 3545.6	797.0 ± 203.5

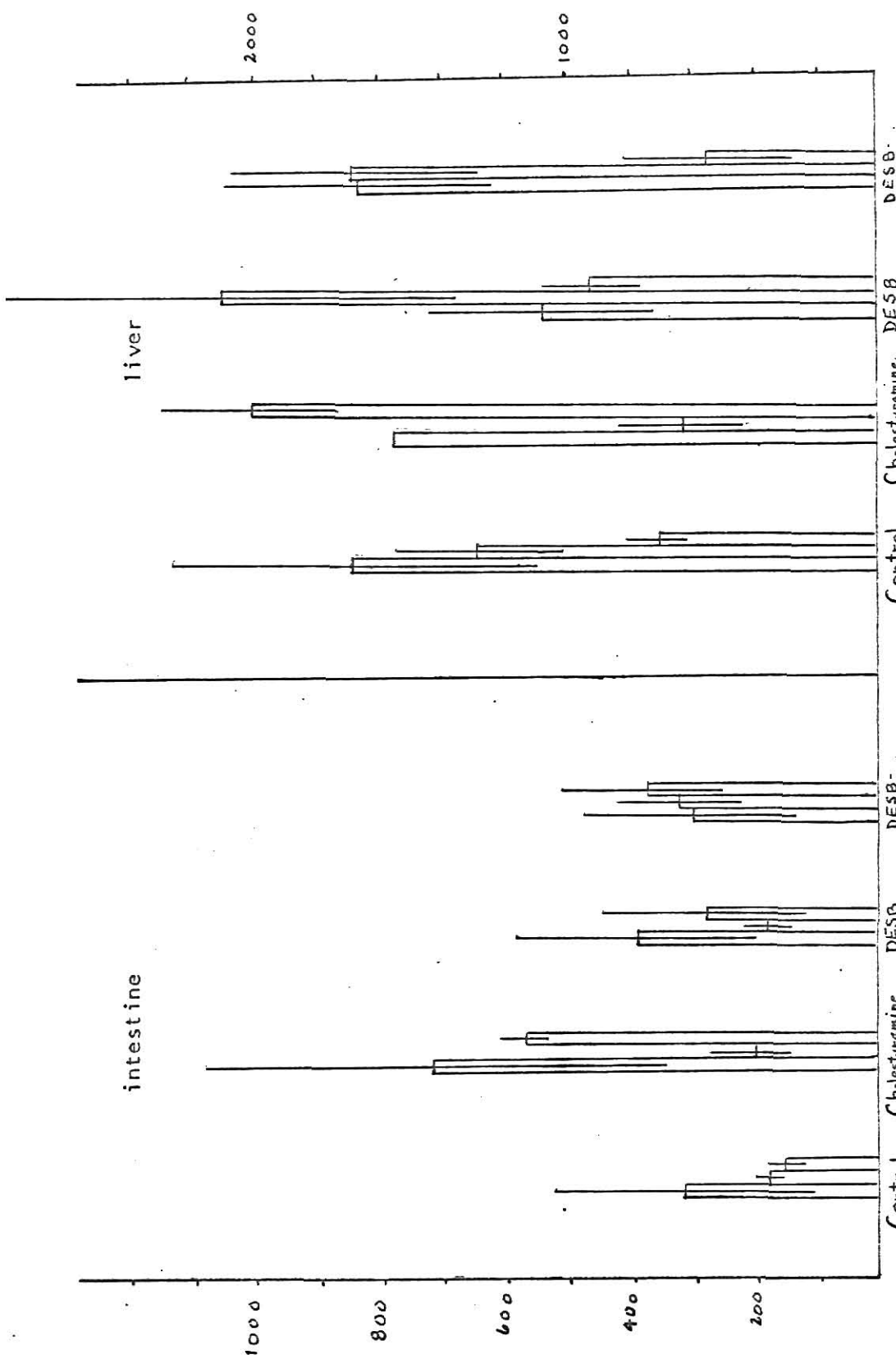


Figure 7. Disintegrations per minute per gram tissue of Δ^7cholestenol in intestine and liver.

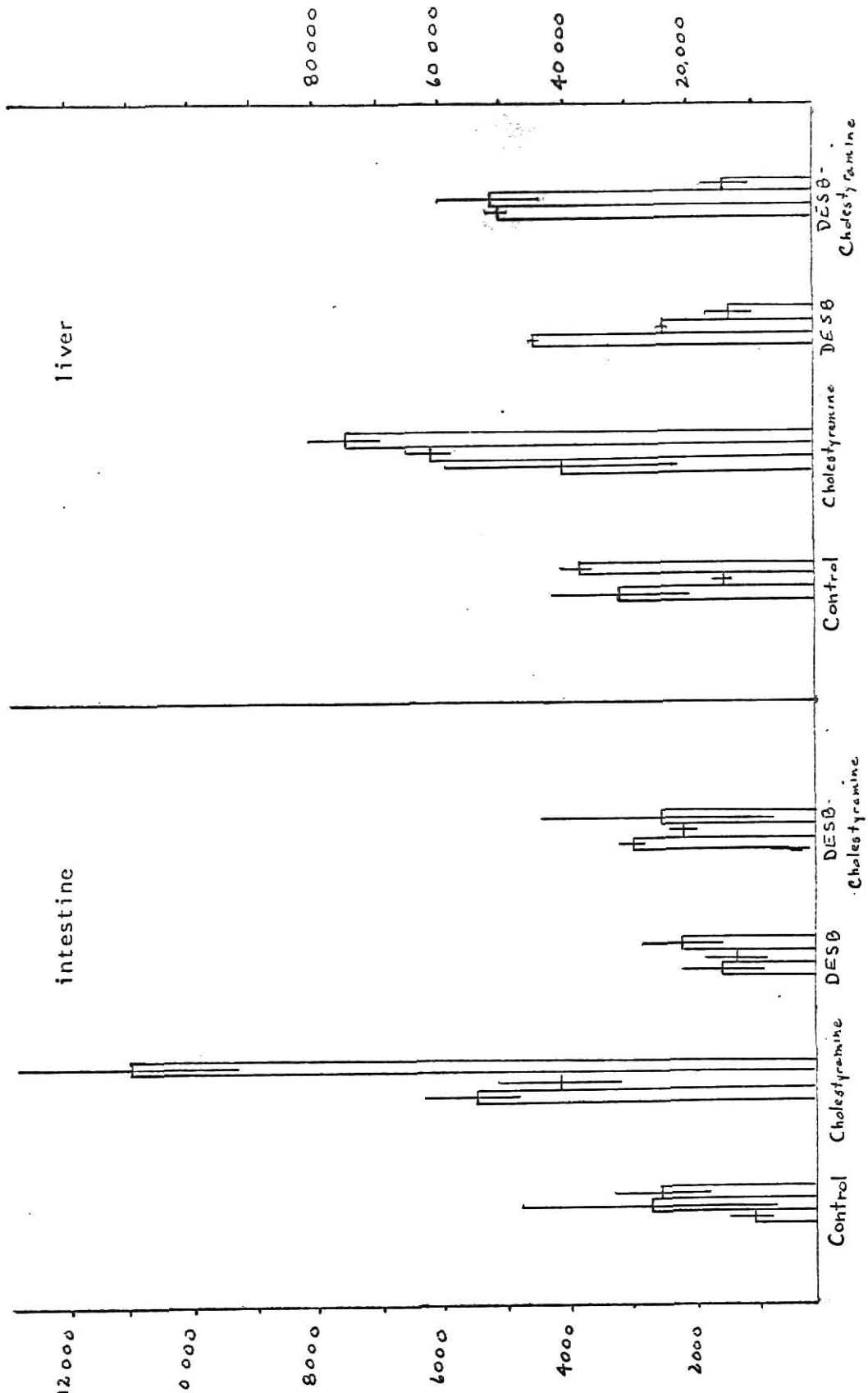


Figure 8. Disintegrations per minute per gram tissue of cholesterol in intestine and liver.

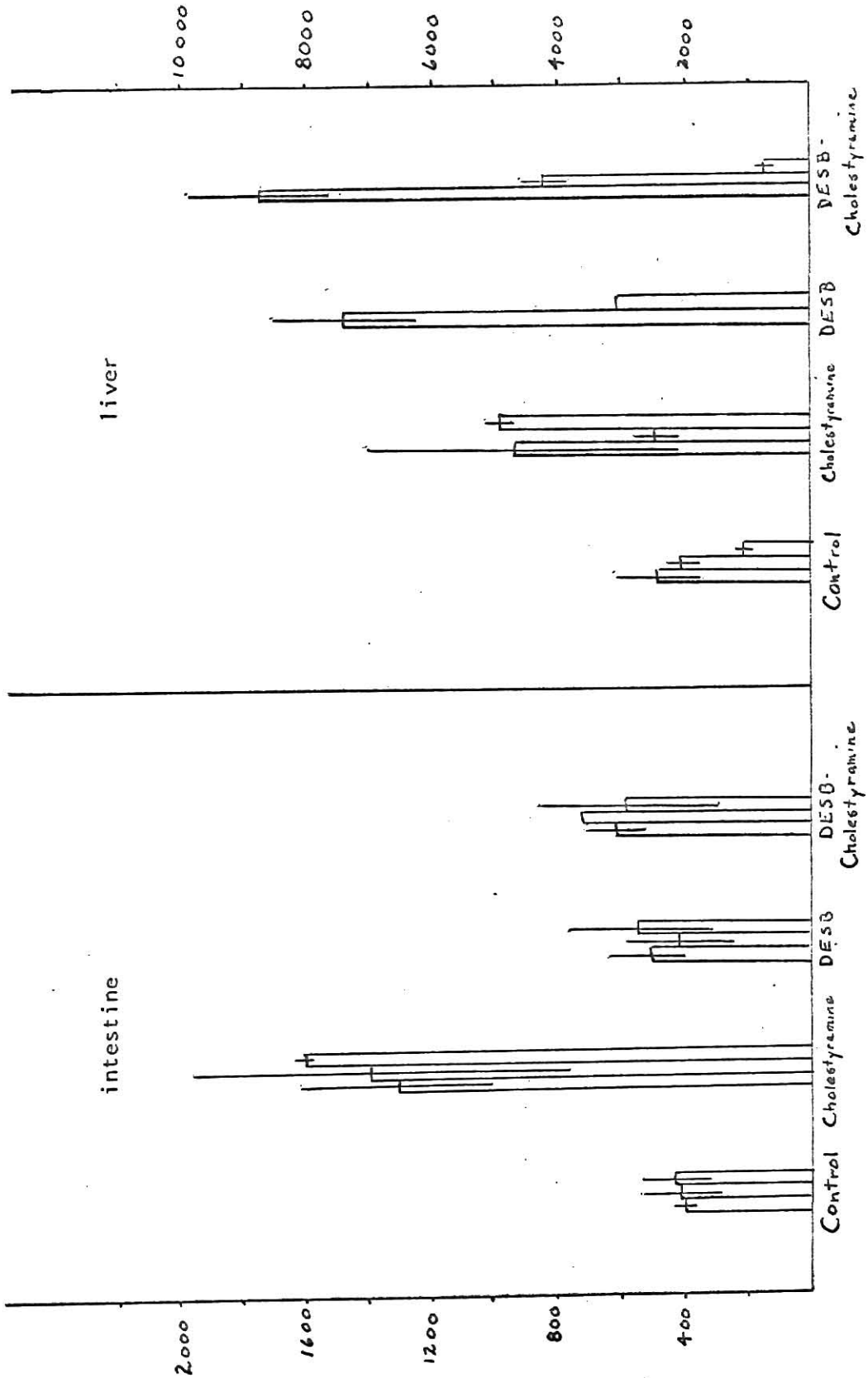


Figure 9. Disintegrations per minute per gram tissue of cholestane (solvent front) in intestine and liver.

than one bird of Group A was sacrificed on any given day, with the three chickens subjected to the same regimen being sacrificed within the span of a week. For example, the three birds being treated with cholestyramine were sacrificed on Monday, Wednesday and Friday of the same week, and the entire schedule for killing the animals extended over approximately four weeks. Three birds of Group B were killed at any one time, the three birds having been subjected to the same regimen of drug treatment. In this manner, all 12 birds of Group B were sacrificed within the time span of approximately one week. As a result of sacrificing a larger number of animals at any given time the possibility of significant variations in the reagents used, particularly the Krebs-bicarbonate buffer, was reduced. Because of the condensed schedule of the Group B birds, the ages of the animals did not vary as much as the Group A birds did.

It was concluded that cholestyramine feeding increased cholesterol synthesizing activity in both the gut and liver. This result was not surprising because cholestyramine removed bile acid from the intestine. This results in no inhibition in intestinal synthetic activity. Since bile acids are requisite for the transport of intestinal cholesterol through the entero-lymphatic system to the liver, this cholesterol is unable to reach the liver. Therefore no feedback inhibition of hepatic cholesterol synthesis occurs and the liver may synthesize greater quantities of cholesterol. Cholestyramine feeding also appears to have increased production of Δ^7-cholestenol in the intestine, and cholestane, i.e., compounds traveling with the solvent front, in both liver and intestinal slices.

The effect of diethylstilbestrol treatment was not so clear cut. It did not cause an increase in the synthetic activity level in either organ. It appears that, at least as far as the synthesis of cholesterol was con-

cerned, it may have had a slight depressing effect on intestinal synthesis. The combination of DESB and cholestyramine gave values that were essentially the same as those obtained in control birds for all compounds isolated from both tissues.

The question now arises as to why DESB treatment did not increase cholesterol synthetic activity in the organs tested. No answer is obvious from the present data. One explanation could be that DESB caused a greater mobilization of lipid stores in the bird. Another possibility was that stilbestrol facilitated greater usage of dietary cholesterol. In such a situation the amount of neutral steroids excreted would be reduced, the lipid level in the serum could rise and there would be no increased synthesis level in the tissues. One would expect, perhaps, a decrease of hepatic cholesterol biosynthesis.

Because the action of the DESB was not clearly elucidated it could be worthwhile to pursue this question in the future. Perhaps a larger daily dose of this hormone would bring about greater changes in synthetic activity levels in an organism. By monitoring the amounts of neutral steroids excreted, insight could be gained as to the mode of action of diethylstilbestrol.

There are a large number of coupounds that affect serum cholesterol levels ranging from antibiotics to hormones to newly synthesized compounds identified only by a series of numbers and letters. As in the past, the efficacy of these drugs is measured by their ability to lower serum cholesterol levels. However, as was shown with cholestyramine treatment, the effect on serum levels is not necessarily accompanied by a similar effect on synthesis at the level of the tissues as can be demonstrated by use of tissue slices. The mode of action of these compounds, in most cases, is not

known. Studies similar to those recently completed, using tissue slices, could be run using tissues from birds treated with such hypocholesterolemic compounds. Another approach would be to use these cholesterol reducing agents with labeled cholesterol precursors in the live animal. Although more unwieldy an experiment than one using tissue slices, this system could give information about in vivo synthesis of sterols and, depending upon the precursor used, information concerning sites of inhibition of cholesterol synthesis.

SUMMARY

The purposes of this study were to explore the effects of diethylstilbestrol and cholestyramine upon the cholesterol synthesizing activities of intestinal and liver slices in the bird, compare the results obtained with those obtained from previous work on serum cholesterol levels and, if possible, suggest an explanation for the results obtained.

Four groups of birds were used; controls; those treated with cholestyramine; those treated with diethylstilbestrol; and those treated with both cholestyramine and diethylstilbestrol. Tissue slices from the intestine and liver were incubated with buffer and C^{14} labeled sodium acetate. The steroids were extracted and separated on thin layer chromatography plates. The spots were scraped into scintillation vials and the radioactivity in each spot determined. The number of disintegrations per minute in each spot was taken as an indication of synthetic activity.

It was found that tissue slices from birds treated with cholestyramine showed an increased rate of incorporation of C^{14} into the cholesterol fraction. The fraction containing Δ^7-cholestenol and the fraction traveling with the solvent front also showed increased incorporation of labeled acetate.

The tissue slices from diethylstilbestrol-treated birds indicated no increase in synthetic activity in either tissue. This estrogen treatment may have, in fact, lowered incorporation of radiotracer to levels below those found in the control animals.

Samples from birds treated with both cholestyramine and diethylstilbestrol showed approximately the same level of incorporation of C^{14}-acetate as did tissue slices from control birds.

An attempt was made to explain the results of this study in light of work done by other experimenters. Plans for future work were also suggested.

REFERENCES CITED

1. Hotta, S. and Chaikoff, I. L., Arch. Biochem. 56, 28 (1955).

2. Gould, R. G., Am. J. Med. 11, 209 (1951).

3. Friedman, M., Byers, S. O. and Michaelis, F., Am. J. Physiol. 164, 789 (1951).

4. Harper, P. V., Neal, W. B. Jr., and Hlavacek, G. R., Metabolism 2, 69 (1953).

5. Srere, P. A., Chaikoff, I. L., Treitman, S. S. and Burstein, L. S., J. Biol. Chem. 182, 629 (1950).

6. Lindsey, C. A. Jr. and Wilson, J. D., J. Lipid Res. 6, 173 (1965).

7. Dietschy, J. M. and Siperstein, M. D., J. Clin. Invest. 44, 1311 (1965).

8. Tomkins, G. M., Sheppard, H. and Chaikoff, I. L., J. Biol. Chem. 201, 137 (1953).

9. Langdon, R. G. and Bloch, K., J. Biol. Chem. 202, 77 (1953).

10. Tomkins, G. M. and Chaikoff, I. L., J. Biol. Chem. 196, 569 (1952).

11. Siperstein, M. D. and Guest, M. J., J. Clin. Invest. 39, 642 (1960).

12. Siperstein, M. D. and Fagan, V. M., J. Biol. Chem. 241, 602 (1966).

13. Frantz, I. D. Jr., Schneider, H. S. and Hinkelman, B. T., J. Biol. Chem. 206, 465 (1954).

14. Bucher, N. L. R., McGarrahan, K., Gould, E. and Loud, A.V., J. Biol. Chem. 234, 262 (1959).

15. Dietschy, J. M., and Siperstein, M. D., J. Lipid Res. 8, 97 (1967).

16. Hutchens, T. T., Van Bruggen, J. T., Cockburn, R. M. and Alest, E. S., J. Biol. Chem. 208, 115 (1954).

17. Jansen, G. R., Zanetti, M. E. and Hutchison, C. F., Biochem. J. 99, 333 (1966).

18. Cayen, M. N., Biochim. Biophys. Acta 187, 546 (1969).

19. Levin, L., Endocrinology 37, 34 (1945).

20. Rosenman, R. H., Friedman, R. and Byers, S. O., Endocrinology 51, 142 (1952).

21. Mukherjee, S. and Bhose, A., Biochim. Biophys. Acta 164, 357 (1968).

22. Lorenz, F. W., Chaikoff, I. L., and Entenman, C., J. Biol. Chem. 126, 763 (1938).

23. Chaikoff, I. L., Lindsay, S., Lorenz, F. W. and Entenman, C., J. Exper. Med. 88, 373 (1948).

24. Dietschy, J. M., Gastorenterology 57, 461 (1969).

25. Treadwell, C. R. and Vahouny, G. V. "Cholesterol Absorption" in C. F. Code (ed.) Handbook of Physiology p. 1407. American Physiological Society, Washington, D. C. (1968).

26. Simmonds, W. J., Hofmann, A. F. and Theodor, E., J. Clin. Invest. 46, 874 (1967).

27. Dietschy, J. M., J. Clin. Invest. 47, 286 (1968).

28. Wilson, J. D. and Reinke, R. T., J. Lipid Res. 9, 85 (1968).

29. Seitz, W. and von Brand, V., Klin. Wochenschr. 39, 891 (1961).

30. Fimognari, G. M. and Rodwell, V. W., Science 147, 1038 (1965).

31. Myant, N. B. and Eder, H. A., J. Lipid Res. 2, 363 (1961).

32. Dietschy, J. M. and Wilson, J. D., J. Clin. Invest. 47, 166 (1968).

33. Huff, J. W., Gilfillin, J. L., and Hunt, V. M., Proc. Soc. Exp. Biol. Med. 114, 352 (1963).

34. Fredrickson, D. S., Loud, A. V., Hinkelman, B. T., Schneider, H. S. and Frantz, I. D. Jr., J. Exp. Med. 99, 43 (1954).

35. Weis, H. J. and Dietschy, J. M., J. Clin. Invest. 48, 2398 (1969).

36. Hashim, S. A. and Vandtallie, T. B., J. Amer. Med. Assoc. 192, 289 (1965).

37. Tennent, D. M., Sietel, H., Zanetti, M. E., Kuron, G. W., Ott, W. H. and Wolf, F. J., J. Lipid Res. 1, 469 (1960).

38. Holmes, W. L., Clin. Med. 77, 41 (1970).

39. Unpublished results.

40. Schneider, D. L., Gallo, D. G., and Sarett, H. P., Proc. Soc. Exp. Biol. Med. 121, 1244 (1966).

41. Gallo, D. G. and Sheffner, A. L., Proc. Soc. Exp. Biol. Med. 120, 91 (1965).

42. Jansen, G. R. and Zanetti, M. E., J. Pharm. Sci. 54, 863 (1965).

43. Sperry, W. M., J. Lipid Res. 4, 221 (1963).

44. Van Lier, J. E. and Smith, L. L., Anal. Biochem. 24, 419 (1968).

THE EFFECTS OF DIETHYLSTILBESTROL AND CHOLESTYRAMINE ON LIPID SYNTHESIS IN THE CHICK AND JAPANESE QUAIL

by

ANNETTE C. REYNOLDS

B. A., West Chester State College, 1967

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1971

ABSTRACT

The effects of diethylstilbestrol and cholestyramine upon sterol synthesis in avian intestinal and liver slices were studied. Tissue slices were incubated with ^{14}C-acetate and buffer. The incorporation of radiotracer into cholesterol and related steroids as measured by liquid scintillation was taken as a measure of synthesizing activity.

It was found that tissue slices from chickens treated with cholestyramine showed an increased rate of incorporation of labeled acetate into the cholesterol fraction. The fraction containing Δ^7-cholestenol and the fraction traveling with the solvent front also showed increased incorporation of labeled acetate.

The tissue slices from diethylstilbestrol-treated birds indicated no increase in synthetic activity in either tissue.

Samples from birds treated with both cholestyramine and diethylstilbestrol showed approximately the same level of incorporation of labeled acetate as did tissue slices from control birds.

An attempt was made to explain the results of this study in light of work done by other experimenters, especially as concerns feedback control in both organs. Plans for future work were also suggested.