incorporation of adenine-8-C^{14} into RNA adenine was higher or unchanged, into RNA guanine lower or unchanged, and into DNA adenine variable; incorporation of thymidine-H^3 into DNA was lower. Normal rabbit serum and immune rabbit serum containing agglutinins to human leukocytes were tested for effects on incorporation of precursors into nucleic acids and protein. Immune serum at a concentration causing macroscopic agglutination of the cells gave results similar to those with normal (non-agglutinating) serum.

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1. Holman, H. R., Deicher, H. R. G., Kunkel, H. G., *Bull. N. Y. Acad. Med.*, 1959, v35, 409.
2. Calabresi, P., Edwards, E. A., Schilling, R. F., *J. Clin. Invest.*, 1959, v38, 2091.
3. Robinson, J. R., *Biochem. J.*, 1949, v45, 68.
4. Williams, A. M., Schilling, R. F., *J. Lab. Clin. Med.*, In Press, 1961.
5. Frenster, J. H., Best, W. R., Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 887.

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Studies on Bile Acids in Rat Systemic Blood. Bile Acids and Steroids 107.* (26610)

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For several decades many attempts have been made to estimate blood bile acid levels (Sobotka(1)). The results of these studies have varied greatly and even during the past 10 years values ranging from 0 to 40 mg per 100 ml plasma have been reported(2). Most values have been obtained using direct colorimetric methods of doubtful specificity. During the last few years chromatographic methods have been applied prior to spectrophotometric or fluorometric measurements(3,4). With these methods no bile acids could be detected in normal human serums. Although bile acids have never been isolated from blood, Carey(5) has observed spots corresponding to bile acids on paper chromatograms of purified human serum extracts. He also found through spectrophotometric methods trihydroxy- and dihydroxycholanic acid levels of 0.14 and 0.08 mg/100 ml respectively(6).

Byers and Friedman have reported the cholate level in rat serum to be 2.1 mg/100 ml(7). Since bile acid metabolism has been studied extensively in the rat we have further investigated the bile acids in the systemic blood of this animal. This work is a continuation of a previous study of the portal bile acids(8).

Experimental. Cholic acid was labelled by exposure to one curie of tritium gas(9) for 14 days in the apparatus described by Bergström and Lindstedt(10). To remove labile tritium the cholic acid was repeatedly evaporated with ethanol-water, then heated for 16 hours at 120°C in 2 N NaOH. After ether extraction of the acidified solution the acid was chromatographed twice with phase system C described below. The cholic acid peak was diluted with twice the weight of inactive cholic acid and recrystallized twice giving a specific activity of about 6.9×10^8 cpm per mg when counted in an infinitely thin layer in a methane gas-flow counter (Frieseke Hoepfner FH 51).

Sprague-Dawley rats weighing 200-300 g were used. 1-2 mg of labelled sodium cholate was administered either orally or intraperitoneally in one or 3 ml of saline respectively. The rats were fed regular stock diet and water *ad lib.* Twenty-four or 48 hours after

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TABLE I. Calculation of Concentration of Cholic Acid and Its Metabolites in Blood of Rats Receiving Tritium Labelled Cholic Acid.

Animal	Cholic acid-T	Equilibration time, hr	cpm × 10^{-6}, intestine	cpm × 10^{-6} in 100 ml blood	Blood "cholic acid," mg/100 ml*
1	1 mg i.p.	24	283	2.42	.128
2	1 " "	24	224	1.24	.083
3	2 " "	48	385	2.17	.085
4	2 " "	48	321	1.80	.084
5	1 mg p.o.	24	373	2.31	.093
6	2 " "	48	420	1.53	.055
7	2 " "	48	318	1.17	.055

* Assuming intestinal cholic acid and its metabolites to be 15 mg(12-15).

administration systemic blood was collected under light ether anesthesia by aortic puncture into a heparinized syringe. A measured volume was added dropwise into 20 volumes of 96% ethanol. Following thorough mixing the volume was diluted to 250 ml with ethanol. The small and large intestines were removed, cut into small pieces and extracted separately by refluxing 3 times for 2 hours in 80% ethanol. The extracts were filtered through glass wool into a volumetric flask and diluted to 1000 ml with ethanol. Radioactivity of all extracts was determined by counting in an infinitely thin layer on aluminium planchets in a Frieseke Hoepfner FH 51 methane gas-flow counter. Ten determinations were made on each sample.

The ethanol extract of the blood was filtered and evaporated. In most cases 5 mg each of cholic and glycocholic acids were added, and the residue extracted with butanol from an acidified water solution(11). After evaporation of the butanol the residue was subjected to reversed phase chromatography with 50% methanol as moving phase and 50% isooctanol-chloroform as stationary phase (phase system C(12)). A 4.5 g column was used. Two ml of the ethanol extract of the small intestine were treated in the same way.

Radioactivity of all the fractions collected from the column was determined and the proportion of different bile acids was calculated from the total radioactivity in the different bands. The activity retained on the column was negligible. The locations of glycocholic and cholic acids were determined by titrations of the fractions with 0.02 N NaOH in methanol.

Results and discussion. The tritiated cholic acid was given intraperitoneally to 4 rats; 2 were killed after 24 hours and 2 after 48 hours. Three rats received the acid orally; one of these was killed after 24 hours and the other 2 after 48 hours. Results of the isotope measurements of the blood and intestinal extracts are given in Table I. Between 5 and 11 ml of blood was obtained from each animal. The distribution of activity between small intestine and large intestine was approximately the same as that found in a previous investigation(13), *i.e.* about 10% was present in the large intestine. Table I shows a calculation of the concentration of labelled bile acids in the blood. The following assumptions have been made in this calculation: 1) The "pool" of cholic acid and its metabolites in the intestine is 15 mg and 2) The labelled bile acids in the rat are completely equilibrated with this unlabelled "pool." From the results of several independent investigations(12-15) it appears acceptable to assume a "cholic acid pool" of 15 mg. This figure has been multiplied by the ratio of radioactivity in 100 ml of blood to radioactivity in intestine, which gives a value for blood cholic acid and metabolites in mg %. The second assumption concerning equilibration of labelled bile acid with the unlabelled pool seems justified from earlier investigations(12). However, to exclude errors due to uneven distribution of the isotope, the labelled acid was given intraperitoneally and orally and was allowed to equilibrate for 24 hours or 48 hours. After intraperitoneal administration an uneven distribution of the isotope between blood and intestine would result in higher specific activity

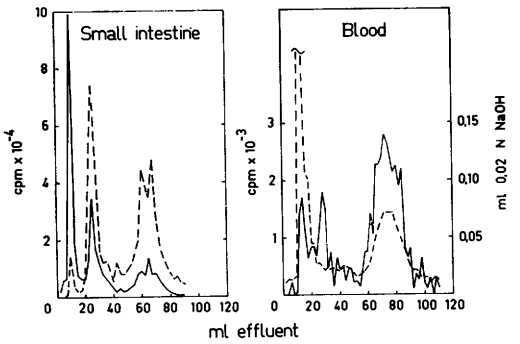

FIG. 1. Chromatograms of labelled compounds in small intestine and blood after administration of tritium labelled cholic acid. Solid line: radioactivity. Broken line: titration. Inactive glycocholic (20-30 ml) and cholic (50-80 ml) acids were added to small intestine, cholic acid (60-90 ml) was added to blood.

of blood bile acids than of intestinal bile acids. The reverse would occur after oral administration of labelled material. Under these circumstances intraperitoneal administration of labelled bile acid would result in higher blood bile acid values than those obtained after ingestion of this compound. In addition this difference would decrease with time. The blood bile acid levels obtained under the various conditions employed (Table I) support the assumption that a rapid equilibration of the labelled cholic acid takes place. The mean value found for cholic acid and its metabolites in whole blood was about 0.08 mg/100 ml. The value for serum cholic acid given by Friedman and Byers(7) is considerably higher but the specificity of the method used by these authors appears doubtful (2).

The nature of the labelled compounds in the blood was studied by reversed phase partition chromatography. Three main peaks of radioactivity were obtained which appeared in the effluent as taurocholic, glycocholic and cholic acids (Fig. 1). Since previous investigations (12) make it improbable that more than traces of other labelled acids could be present in these peaks, their identity was established only by chromatography together with inactive carrier compounds. Total activity in the 3 peaks was counted as 100% and the proportion between the peaks was calculated (Table II). The minute amounts of radioactivity retained in the columns represented incomplete elution of the compounds mentioned above and the presence of free deoxycholic acid could not be demonstrated.

A small percentage of free bile acids has previously been shown to be present in rat portal blood(8). Most of these acids represented metabolites formed in the cecum from the tritium labelled cholic acid administered, mainly deoxycholic acid. The surprisingly large percentage of free bile acids in combination with the absence of free deoxycholic acid in systemic blood made it improbable that these acids should have come from the cecum. It is highly improbable that some of the originally injected cholic acid should remain unconjugated after 24 hours and even more so after 48 hours. Therefore a chromatographic analysis was made of the labelled bile acids in the small intestine which contains the major part of the bile acid pool and that is the main source of portal bile acids. Contrary to previous results(12) fairly large amounts of free bile acids were found (Fig. 1 and Table II). Paper chromatography of small intestinal contents also showed spots corresponding to the labelled acids. The

TABLE II. Chromatographic Analysis of Labelled Bile Acids in Small Intestine and Blood of Rats Receiving Tritium Labelled Cholic Acid.

	Labelled bile acids in small intestine					Labelled bile acids in blood				
	Conjugated			Free	Ratio,	Conjugated			Free	Ratio,
Rat	Tau	Gly	Total		conj/free	Tau	Gly	Total		conj/free
1 i.p.	—	—	—	—	—	10	14	24	76	.3
2 "	—	—	—	—	—	21	26	47	53	.9
3 "	61	7	68	32	2.1	7	3	10	90	.1
4 "	45	20	65	35	1.9	20	6	26	74	.4
5 p.o.	80	10	90	10	9.0	57	11	68	32	2.1
6 "	82	9	91	9	10.1	42	13	55	45	1.2
7 "	40	32	72	28	2.6	12	18	30	70	.4

reason for this discrepancy is not known but it probably results from differences in the intestinal flora of the rats used in the two investigations. When a bile fistula is made on a rat from the strain used in this investigation only conjugated bile acids are excreted, mostly turocholic acid. This is in agreement with previous investigations.

The distribution of radioactivity between the 3 main bile acids found in the small intestine and blood is shown in Table II. The proportion of unconjugated bile acids is much higher in the blood than in the small intestine. The 2 rats (No. 5 and 6) having only small amounts of free bile acids in the small intestine also have a definitely lower percentage of free acids in the blood than the other animals. The results seem to indicate a preferential removal by the liver of the conjugated bile acids present in the portal blood.

Summary. Rats were given tritium labelled cholic acid intraperitoneally or orally and killed by exsanguination after 24 or 48 hours. The concentration of "total cholic acid" in whole blood was calculated from the distribution of isotope in intestine and blood and previous figures for intestinal "cholic acid." A value of about 0.08 mg/100 ml was found. Chromatographic analysis showed that more unconjugated cholic acid was present in the systemic blood than in the small intestine.

1. Sobotka, H., *Physiological Chemistry of the Bile,* Baltimore, Williams and Wilkins, 1937.
2. Mac Intyre, I., Wootton, I. D. P., *Ann. Rev. Biochem.,* 1960, v29, 635.
3. Rudman, D., Kendall, F. E., *J. Clin. Invest.,* 1957, v36, 530.
4. Osborn, E. C., Wootton, I. D. P., Da Silva, L. C., Sherlock, S., *Lancet,* 1959, vII, 1049.
5. Carey, J. B., Jr., *Science,* 1956, v123, 892.
6. ———, *J. Clin. Invest.,* 1958, v37, 1494.
7. Byers, S. O., Friedman, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 425.
8. Olivecrona, T., Sjövall, J., *Acta Physiol. Scand.,* 1959, v46, 284.
9. Wilzbach, E. E., *J. Am. Chem. Soc.,* 1957, v79, 1013.
10. Bergström, S., Lindstedt, S., *Acta Chem. Scand.,* 1957, v11, 1275.
11. Norman, A., *Acta Physiol. Scand.,* 1954, v32, 1.
12. Norman, A., Sjövall, J., *J. Biol. Chem.,* 1958, v233, 872.
13. Gustafsson, B., Norman, A., Sjövall, J., *Arch. Biochem. Biophys.,* 1960, v91, 93.
14. Portman, O. W., Murphy, P., *ibid.,* 1958, v76, 367.
15. Eriksson, S., *Acta Physiol. Scand.,* 1960, v48, 439.

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Application of Physical Concepts to Dose-Response Relationships of Mixed Estrogens and Mixed Androgens. A Mathematical Analysis.* (26611)

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The application of physical concepts to biological responsiveness with a view toward achieving a better understanding of biological phenomena has intrigued many workers in the field. We originally reported(1) on the biological responsiveness to topical application of varying mixtures of androsterone and dehydroepiandrosterone to chick combs. We recently reported(2) the results of injecting mixtures of estrone, estriol and estradiol 17β on biological response of the uteri of immature rats. Our results and discussions were based on conventional dose-response relationships. Our present aim was to see if our data would lend themselves to the concept of surface adsorption as set forth in the Langmuir(3) adsorption isotherm, especially since topical application involves direct ap-

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