"fried egg" or "nippled" colonies produced by non-Eaton oral PPLO. The distinctive colonial morphology of Eaton PPLO may ultimately prove useful for its presumptive identification.

1. Liu, C., Eaton, M. D., Heyl, J. T., *J. Exp. Med.*, 1959, v109, 545.

2. Chanock, R. M., Mufson, M. A., Bloom, H. H., James, W. D., Fox, H. H., Kingston, J. R., *J.A.M.A.*, 1961, v175, 213.

3. Chanock, R. M., Rifkind, D., Kravetz, H. M., Knight, V., Johnson, K. M., *Proc. Nat. Acad. Sci.*, 1961, v47, 887.

4. Eaton, M. D., *Handbuch Virusforsch.*, 1950, v2, 87.

5. ———, PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 24.

6. ———, *Antibiotics Annual 1954-1955*, Medical Encyclopedia, Inc., New York, 1046.

7. Chanock, R. M., Hayflick, L., Barile, M. F., *Proc. Nat. Acad. Sci.*, 1962, v48, 41.

8. Liu, C., *J. Exp. Med.*, 1957, v106, 455.

9. Chanock, R. M., Fox, H. H., James, W. D., Bloom, H. H., Mufson, M. A., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 371.

10. Clark, H. W., Fowler, R. C., Brown, T. M., *J. Bact.*, 1961, v81, 500.

11. Kingston, J. R., Chanock, R. M., Mufson, M. A., Hellman, L. P., James, W. D., Fox, H. H., Manko, M. A., Boyers, J., *J.A.M.A.*, 1961, v176, 118.

12. Mufson, M. A., unpublished.

13. Forsyth, B., Bloom, H. H., unpublished.

14. Piercy, S. E., *Ann. N. Y. Acad. Sci.*, 1960, v79, 665.

15. Adler, H. E., *ibid.*, 1960, v79, 703.

Received April 2, 1962. P.S.E.B.M., 1962, v110.

C^{14}-Labeled Streptococcal Hyaluronic Acid in the Guinea Pig.* (27576)

RUSSELL S. JONES AND E. VIRGIL HOWELL
Department of Pathology, University of Utah College of Medicine, Salt Lake City

Synovitis has been produced in guinea pigs (1) by polysaccharides and polysaccharide complexes of gram-negative bacteria. Some of the exudative material in the joint space apparently consisted of masses of hyaluronic acid and protein(2). Little is known concerning the local production and resorption of hyaluronic acid in synovial fluid. A reasonable assumption would be that the half-life of hyaluronic acid in synovium would be similar to that of rabbit skin–namely, 2 to 3 days (3,4). As an approach to the study of synovial hyaluronic acid, the fate of parenterally injected C^{14}-labeled streptococcal hyaluronic acid in the guinea pig has been investigated. As might be expected with a linear polymer of N-acetyl glucosamine-glucuronic disaccharide, streptococcal hyaluronic acid appeared to be identical with that of animals (5), although a lower content of uronic acid has been reported(6).

Methods and materials. C^{14}-labeled hyaluronic acid was obtained from Streptococcus, Group A 111 grown in 2.5% Difco heart infusion broth containing 1% glucose, 0.1% sodium acetate and 6.3 millicuries of sodium acetate-1-C^{14} per liter. During the 30-hour period of growth at 37°C, the culture was neutralized at intervals with sodium hydroxide. The bacterial cells were removed by centrifugation and the crude hyaluronic acid was precipitated from the culture media by addition of 4 volumes of 95% ethanol saturated with potassium acetate according to the method of Pike(7). The precipitated potassium hyaluronate was washed 4 times with 95% ethanol and twice with absolute ethanol. The hyaluronic acid was then purified by the procedure of Roseman *et al.*(6) with removal of protein by phosphomolybdic acid, precipitation of hyaluronic acid from aqueous solution with 3 volumes of acetone and treatment with Amberlite IR-120. The final product was again precipitated as potassium hyaluronate, then washed 4 times with 95% ethanol and twice with ether. The uronic acid content was determined by procedure of Dische(8) and glucosamine content by the

* Supported by research grant from The National Foundation.

Elson-Morgan reaction(9). Paper electrophoresis was carried out using veronal buffer, pH 8.6, Γ/2, 0.075, with localization of labeled hyaluronic acid on electrophorograms by means of a recording isotope rate-meter. Relative viscosity of 0.1% solution of hyaluronate in 0.15 M sodium chloride was determined in an Ostwald viscosimeter having a 0.15 M sodium chloride flowtime of 79.4 seconds at 37°C.

Guinea pigs of either sex and weighing 180 to 250 g were given by ear vein 0.5 mg (per 100 g body weight) labeled hyaluronate prepared as a 0.1% solution in 0.15 M sodium chloride. Animals were placed in closed containers, supplied with oxygen by a demand regulator, $C^{14}O_2$ collected in 2 N NaOH agitated with a magnetic stirrer, and C^{14} content determined from barium carbonate as previously described(10). Urine was collected at intervals and C^{14} content determined. After cooling to 4°C the urinary sediment was removed by centrifugation, and 4 volumes cold 95% ethanol containing 1% potassium acetate added to the supernatant. The resulting precipitate was separated by centrifugation, redissolved in water and again precipitated with ethanol and potassium acetate. The C^{14} content and electrophoretic mobility of this ethanol-insoluble, water-soluble material in urine and of the freshly voided urine was determined by procedures indicated above. Since the urine was found to contain more glucuronic acid than attributable to the C^{14} content as hyaluronic acid, the urine was concentrated, electrophoresis carried out, and the area of C^{14} content corresponding to hyaluronic acid mobility eluted. Glucuronic acid content of the eluate was compared to that of a group of similar electrophorograms eluted from the alcohol precipitates of the urine and from the original streptococcal hyaluronic acid.

The C^{14} content in various tissues, plasma and erythrocytes of animals killed at various intervals after intravenous injection of hyaluronic acid was determined(10). The femorotibial knee joints were washed by injection and withdrawal of 0.1 ml of 0.15 M sodium chloride or by rinsing the carefully opened joint space. Blood was removed by cardiac puncture into syringes moistened with heparin and C^{14} localization in plasma protein was quantitated planimetrically from linear recordings of the electrophorograms. Labeled streptococcal hyaluronic acid was added to pooled, heparinized fresh guinea pig plasma in amounts comparable to that found in the plasma of injected animals at 1 and 3 hours. After one hour of incubation of plasma and hyaluronic acid at room temperature, paper electrophoresis was carried out at the same temperature. The relative distribution of plasma proteins was estimated densitometrically from duplicate electrophorograms stained with bromphenol blue. The electrophoretic mobilities on paper of unlabeled glucosamine and N-acetylglucosamine were determined by spraying with Elson-Morgan reagents and of glucose and glucuronic acid by use of p-anisidine phosphate spray(11); these substances as well as acetate were also identified by C^{14} labeling. The labeled hyaluronic acid was hydrolyzed with 1 N HCl at 98° for 4 and 12 hours. Paper electrophoresis of the neutralized hydrolysate was carried out by the technics indicated above except that the electrophorograms were dried briefly at room temperature prior to detection of the C^{14}-containing zones. Steam distillation of the hydrolysate was performed and radioactivity in the sodium salt of volatile fraction determined.

The various C^{14}-containing zones of a group of wet electrophorograms of the hydrolysates were eluted with weak acid, concentrated and applied, both directly as well as after drying and extraction with pyridine, to paper for chromatography in butanol-acetic acid-H_2O (4:1:5) and liquid phenol-NH_4OH (10:1). Original hydrolysates, labeled and unlabeled glucuronic acid, glucose, acetate, glucosamine, N-acetyl glucosamine, were chromatographed in the same systems. The volatile C^{14} compound of high electrophoretic mobility was recovered by elution and its hydroxamate chromatographed with reference hydroxylamine derivatives of formic acid and unlabeled and labeled acetic acid in amyl alcohol-acetic acid-H_2O (4:1:5)(11). Detection was by either ferric chloride spray or by isotopic content.

The C^{14}-containing areas on the dried chromatograms were eluted with water, concentrated, and electrophoretic mobilities compared with those of known compounds, hyaluronic and the hydrolysates.

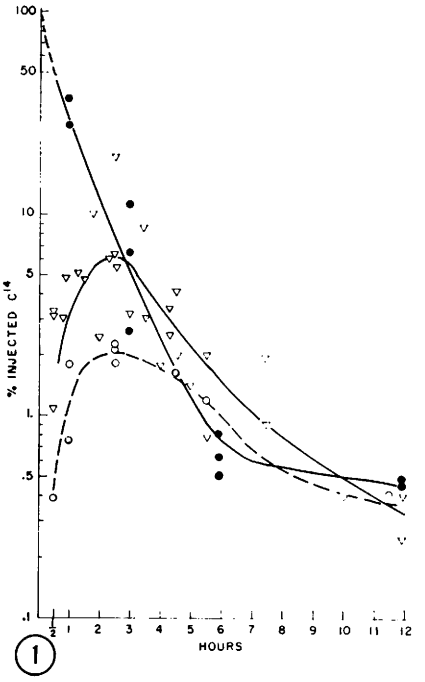
Results. The relative viscosity of the labeled hyaluronic acid was 3.1 and addition of testicular hyaluronidase resulted in immediate reduction of viscosity while addition of hyaluronidase inhibitor (Worthington) together with hyaluronidase prevented this reduction in viscosity.

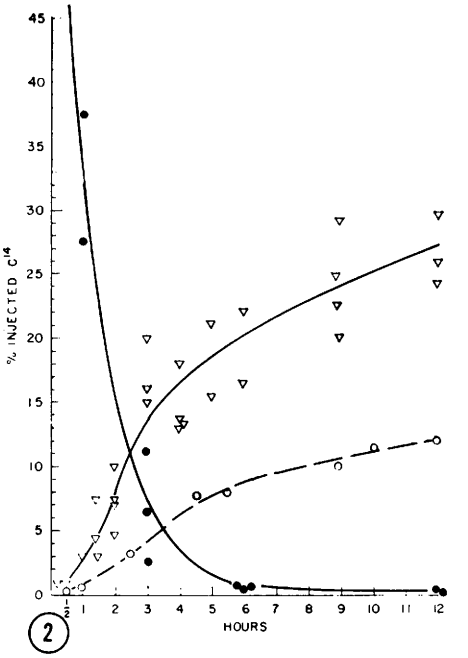
When freed of potassium by treatment with the ion exchange resin, C^{14}-labeled hyaluronic acid had a specific activity of 4.25 μc/mg. Glucuronic acid was 42% of hyaluronic acid by weight and 22.8% of the C^{14}; N-acetyl glucosamine content was 44% by weight and 77.1% of the isotope. The acetyl group, readily freed from N-acetyl glucosamine upon acid hydrolysis, was recovered by steam distillation and by elution of electrophoretic areas and further identified by chromatographic mobility of its hydroxylamine derivative. Almost 75% of the C^{14} in the hyaluronic acid was in the acetyl group. After removal of the acetyl group only 2.6% of the C^{14} was present in the glucosamine.

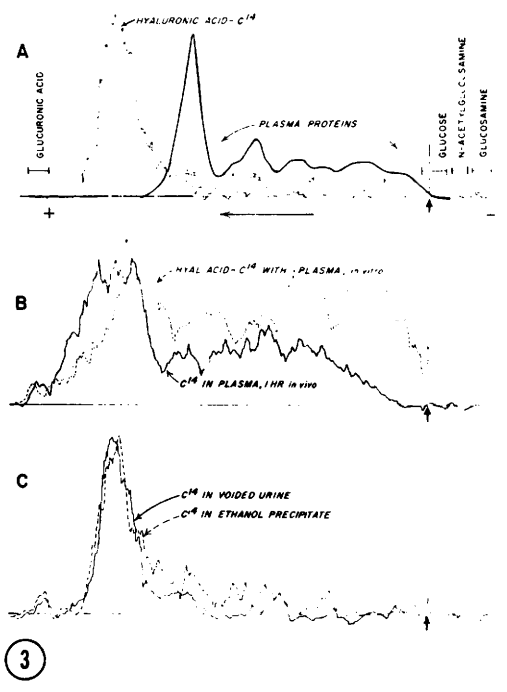
C^{14}-labeled streptococcal hyaluronic acid, after intravenous injection, rapidly disappeared from the plasma of the guinea pig (Fig. 1, 2, 4). Two distinct exponential clearance rates of C^{14} were noted: an initial, rapid clearance rate during the first 6 hours (Fig. 1), followed by a slower rate lasting several days (Fig. 4). A considerable amount of the C^{14} in the plasma 1 to 6 hours after intravenous injection of labeled hyaluronic acid had electrophoretic mobility similar to that of the injected material, which moved as a single peak (Fig. 3). Under the conditions of paper electrophoresis procedure employed, a negligible amount of the isotope moved with the albumin. The small amount of C^{14} remaining in the plasma 9 hours or more after injection of labeled hyaluronic acid was insufficient for satisfactory isotopic localization in the electrophorograms. In comparison to the *in vivo* plasma for the first 6 hours after injection, the labeled hyaluronic acid added in comparable quantity to plasma *in vitro* showed relatively more binding to the globulins and a pronounced pre-albumin peak as well as a prominent zone of distribution near the expected hyaluronic acid peak (Fig. 3B). There was no visible protein staining with bromphenol blue in the pre-albumin zone. The acetic acid solution used in the staining of proteins on the electrophorograms eluted nearly all of the radioactivity beyond the albumin front while little was removed from the globulin zones.

The rapid clearance of hyaluronic acid from the plasma was accompanied by increasing rates of urinary excretion and of respiratory $C^{14}O_2$ loss during the first 2 or 3 hours, followed by a comparable decline (Fig. 1). During the first 12 hours, the cumulative C^{14} loss in the urine and respired CO_2 was 27 and 12% respectively (Fig. 2). The C^{14} in urine had the same electrophoretic mobility as the original hyaluronic acid (Fig. 3C). Between 95 and 100% of the C^{14} in the urine was precipitated with ethanol and potassium acetate. On the basis of glucuronic acid content, the specific activity of this ethanol-insoluble, water-soluble material with electrophoretic mobility of hyaluronic acid, was 48% of that of the original hyaluronic acid. Although only 39% of the injected C^{14} was lost in the urine and respired CO_2, the relatively little C^{14} found in the tissues diminished less rapidly than C^{14} in the plasma (Fig. 4). Almost no C^{14} was found in the synovial fluid, or vitreous humor and little C^{14} was found in erythrocytes or leukocytes washed with 0.15 M sodium chloride. On the basis of tissue concentration, the greatest amount of C^{14} was found in the liver, spleen and ovaries. Intermediate concentrations of isotope were present in salivary glands, skeletal muscle, fat, pancreas, testes, thymus and myocardium.

Comments. Streptococcal hyaluronic acid, injected intravenously, was rapidly cleared from the plasma, excreted in the urine and partially metabolized with loss of C^{14} in respired CO_2. Intravenously injected macromolecular substances usually have been found to have a rapid initial exponential decrease followed by a slower clearance from the plasma but both phases of hyaluronic acid clearance were rapid in comparison to that of







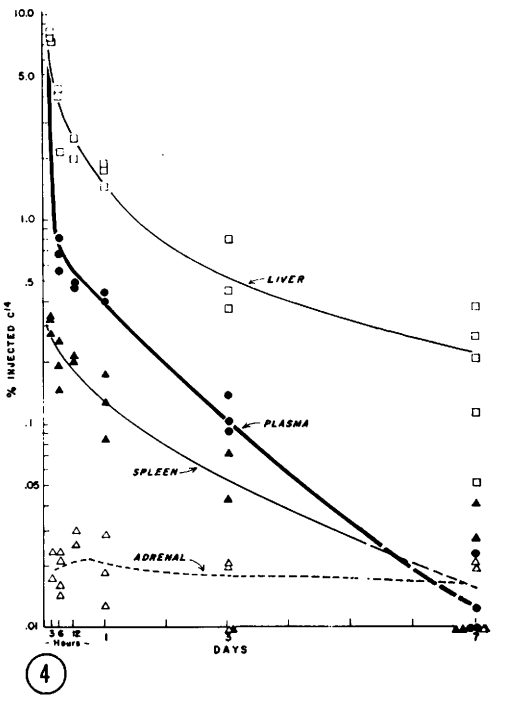


FIG. 1. Clearance of intrav. inj. C^{14}-labeled streptococcal hyaluronic acid from plasma (●—●) of guinea pig in comparison to % per hr excreted in urine (▽—▽) and % per hr lost as respired $C^{14}O_2$ (○- -○); semilogarithmic plot.

FIG. 2. Plasma clearance of intrav. inj. labeled hyaluronic acid (●—●) and cumulative loss as hyaluronic acid in urine (▽—▽) and as respired $C^{14}O_2$ (○- -○); linear plot.

FIG. 3. Paper electrophoretic mobilities of C^{14}-labeled streptococcal hyaluronic acid in plasma and in urine. A) Relative mobilities of plasma proteins, labeled hyaluronic acid, glucu-

ronic acid, glucose, N-acetyl glucosamine and glucosamine. B) Comparison of C^{14}-labeled streptococcal hyaluronic acid in guinea pig plasma 1 hr after intrav. inj., 36.5 μg/ml (——, *in vivo*), with labeled hyaluronic acid to guinea pig plasma, 50 μg/ml (– – –, *in vitro*). C) Electrophoretic mobilities of C^{14} in voided urine and of alcohol-precipitable material in the same urine.

FIG. 4. Percent of inj. C^{14} present in plasma, liver, spleen, and adrenal at different intervals following inj. of labeled streptococcal hyaluronic acid.

Klebsiella pneumoniae polysaccharide, for example(10). Although the rapid initial disappearance of streptococcal hyaluronic acid from plasma may be attributable in part to distribution in extracellular fluids and tissues, with dilution in the abundant hyaluronic acid of connective tissue, C^{14} was almost absent from synovial fluid and skin.

Binding of streptococcal hyaluronic acid to plasma protein may have significant roles in the clearance from plasma, especially during the second phase beginning at about the 6th hour, when 1 μg of labeled hyaluronic acid per ml plasma was present. Electrophoretic studies, however, suggested a much higher protein-binding capacity: 1 hour after injection only 10 to 15 μg per ml or 30 to 40% of the circulating labeled hyaluronic acid appeared bound to plasma protein (Fig. 3B). However, electrophoretic studies may not reflect actual relationship between hyaluronic acid and plasma proteins *in vivo* since a) the relative amount of "free" to "bound" hyaluronic acid was similar at 1, 3, and 6 hours after injection, despite marked reduction in total quantity and, b) relatively more hyaluronic acid was bound to plasma protein *in vitro* than *in vivo* (Fig. 3B) c) electrical potential, *per se,* would have reduced covalent bonding between hyaluronic acid and protein.

In contrast to the rapid initial clearance of hyaluronic acid from plasma, maximal rates of respiratory $C^{14}O_2$ and urinary C^{14} loss were not attained until the 2nd and 3rd hours. The delay in attainment of maximal urinary excretion was not attributable to retention of hyaluronic acid in kidney or urinary bladder, since only low concentrations of C^{14} were found in the kidney and since comparable quantities of hyaluronic acid were present in urinary bladders of sacrificed animals and in voided urine during the first 3 hours. Perhaps some depolymerization of hyaluronic acid preceded either excretion or metabolic degradation. The uptake of label into reticuloendothelia or other tissue cells, although rapid, may have been followed by a slower phase of degradation. If the 2 constituents of hyaluronic acid, N-acetylglucosamine and glucuronic acid, were metabolized directly, then maximal respiratory $C^{14}O_2$ loss would have been expected during the 1st hour, as noted with relatively metabolically inert glucuronic acid(12) or sooner, as noted with glucosamine(13).

In the present study, approximately 75% of the label was incorporated biosynthetically into the acetyl groups of streptococcal hyaluronic acid. It is possible that the acetyl group of injected streptococcal hyaluronic acid(14) was exchanged more rapidly than that reported for tissue hyaluronic acid (4) and thereby afforded a ready source of the respired $C^{14}O_2$. Although C^{14} acetate was recovered from the hydrolysates of the macromolecular material excreted in the urine, some deacetylation or acetyl exchange could have occurred. However, the increased hexuronic acid to C^{14} ratio of the hyaluronic acid recovered from urine by either precipitation or electrophoresis could have been due to admixture with endogenous material as well as to loss of labeled acetyl moiety.

If hyaluronic acid from streptococcal and tissue sources are handled similarly by the animal, then hyaluronic acid, after gaining access to the blood stream, is rapidly eliminated by urinary excretion, organ uptake and degradation, but not by entry into connective tissue or synovial fluid.

Summary. Streptococcal hyaluronic acid was biosynthetically labeled with C^{14} from acetate-1-C^{14}. Approximately 75% of the label was in the acetyl of N-acetyl glucosamine. After intravenous injection, C^{14}-labeled streptococcal hyaluronic acid was rapidly cleared from the plasma. Electrophoresis disclosed some binding of hyaluronic acid to plasma proteins. The maximal rate of respiratory $C^{14}O_2$ loss and urinary excretion of

hyaluronic acid did not occur until the 2nd and 3rd hour following intravenous injection. Only traces of intravenously injected hyaluronic acid appeared in the synovial space, skin or vitreous humor.

1. Jones, R. S., Carter, Y., *Arch. Path.*, 1957, v63, 472.
2. Jones, R. S., Mayne, Y. C., *ibid.*, 1958, v65, 247.
3. Schiller, S., Mathews, M. B., Goldfaber, L., Ludowieg, J., Dorfman, A., *J. Biol. Chem.*, 1955, v212, 531.
4. Schiller, S., Mathews, M. B., Cifonelli, J. A., Dorfman, A., *ibid.*, 1956, v218, 139.
5. Whistler, R. L., Olson, E. J., *Adv. Carbohydrate Chem.*, 1957, v12, 299.
6. Roseman, S., Moses, F. E., Ludowieg, J., Dorfman, A., *J. Biol. Chem.*, 1953, v203, 213.
7. Pike, R. M., *J. Inf. Dis.*, 1948, v83, 1.
8. Dische, Z., *J. Biol. Chem.*, 1947, v167, 189.
9. Gardell, S., *Methods Biochem. Analysis*, 1958, v6, 309.
10. Jones, R. S., Carter, Y., *Arch. Path.*, 1957, v63, 484.
11. Lederer, E., Lederer, M., in *Chromatography*, 2nd ed., 1957, Elsevier Publ. Co.
12. Douglas, J. F., King, C. G., *J. Biol. Chem.*, 1953, v203, 889.
13. Boas, N. F., Foley, J. B., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 454.
14. Dorfman, A., Roseman, S., Moses, F. E., Ludowieg, J., Mayeda, M., *J. Biol. Chem.*, 1955, v212, 583.

Received April 30, 1962. P.S.E.B.M., 1962, v110.

In vitro Formation of Deoxycholic and Lithocholic Acid by Human Intestinal Microorganisms.*† (27577)

ARNE NORMAN AND MARY S. SHORB‡
Department of Clinical Chemistry, Stockholms Läns Centrallasarett, Danderyd, Sweden

Elimination of the 7-hydroxysubstituent of the bile acids by intestinal microorganisms is a common reaction occurring in many species (1) and has been reproduced *in vitro* by a mixture of anaerobic intestinal microorganisms from the rat(2). Samuelsson has demonstrated that the reaction mechanism is a dehydration to a Δ^6-derivative, followed by reduction(3).

Human bile contains 3 main bile acids, cholic, chenodeoxycholic and deoxycholic acid, with a mean ratio of 1.1:1.0:0.6(4). Cholic and chenodeoxycholic acids are formed directly from cholesterol in the liver (1). Due to the action of microbial enzymes the 7-hydroxyl group of cholic acid is removed and the "secondary" bile acid deoxycholic acid is formed(5). Contrary to the rapid transformation of cholic acid, chenodeoxycholic acid is transformed slowly during the enterohepatic circulation. Three days after administration of chenodeoxycholic acid-24-C^{14} only 3% of the isotope in the human bile was recovered as lithocholic acid(6). Although definitive proof is lacking, investigation so far indicates that deoxycholic and lithocholic acids are not metabolized by the human liver to a significant extent(6,7). The different concentration of deoxycholic and lithocholic acids in human bile, therefore, might be explained by a difference in ability of the intestinal flora to remove the 7-hydroxyl group from cholic and chenodeoxycholic acid or by a difference in accessibility for absorption of the "secondary" bile acids.

The aim of the present investigation was to study the formation of deoxycholic and lithocholic acid in subcultures of mixed human intestinal microorganisms.

Experimental. The 24-C^{14}-labeled cholic, deoxycholic and chenodeoxycholic acids were prepared according to Bergström *et al.*(8).

Labeled bile acids as sodium salts dissolved in 80% ethanol were added to thick walled centrifuge tubes (inside diameter 13 mm, height 100 mm). After evaporation, the

* Bile acids and steroids 122.

† Supported by research grants from Statens Medicinska Forskningsrad and Svenska Livförsäkringsbolags nämnd för medicinsk forskning.

‡ On sabbatical leave from Poultry Husbandry Dept., Univ. of Maryland.