nemia level. Observation of single patients seems to confirm this statistical finding; patient No. 35, for instance, who showed the highest phenylketonemic level, was a voracious eater, while patients No. 15, 33 and 34, who showed low phenylketonemia, were "feeding problems" and consumed limited amounts of food. The observed correlations for I.Q. and phenylketonemia may then depend simply on the fact that in this series, subjects with high I.Q. usually show better eating habits than low I.Q. patients.

Summary. Phenylpyruvic acid was demonstrated in the blood of 39 patients affected with phenylpyruvic oligophrenia. The values ranged from 0.31 to 1.78 mg per 100 ml of plasma. A significant increase of phenylketonemia was observed following ingestion of DL-phenylalanine, the D form causing considerably greater increase than the L isomer. Feeding of phenylpyruvic acid resulted in a sharp increase of phenylketonemia. The significance of these findings is briefly discussed.

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Nature of the Metabolic Products of C^{14}-Cholesterol Excreted in Bile and Feces.* (19999)

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It has been generally considered that cholesterol is eliminated from the body principally as coprosterol, dihydrocholesterol and cholesterol, and that their excretion—or the excretion of their precursors—takes place across the intestinal wall(1-3). Bile has come to be regarded as a relatively minor path for the elimination of cholesterol or of its products by metabolism(4). Recent studies carried out with cholesterol-4-C^{14} have revealed, however, that this view is no longer tenable, for approximately 90% of the C^{14} of administered cholesterol-4-C^{14} is recoverable in bile. Furthermore, the nonsaponifiable fraction, which includes coprosterol and dihydrocholesterol as well as cholesterol, does not amount to more than 25% of the total C^{14} found in either bile or feces (5). Thus, by far the major part of the administered C^{14} is eliminated as saponifiable materials. It seemed likely that these excretion products are bile acids. Supporting evidence for this view is presented here.

Treatment of rats. Male Long-Evans rats, weighing between 250 and 300 g, were lightly anesthetized with ether. One mg of cholesterol-4-C^{14} containing 3 x 10^6 c.p.m. dissolved

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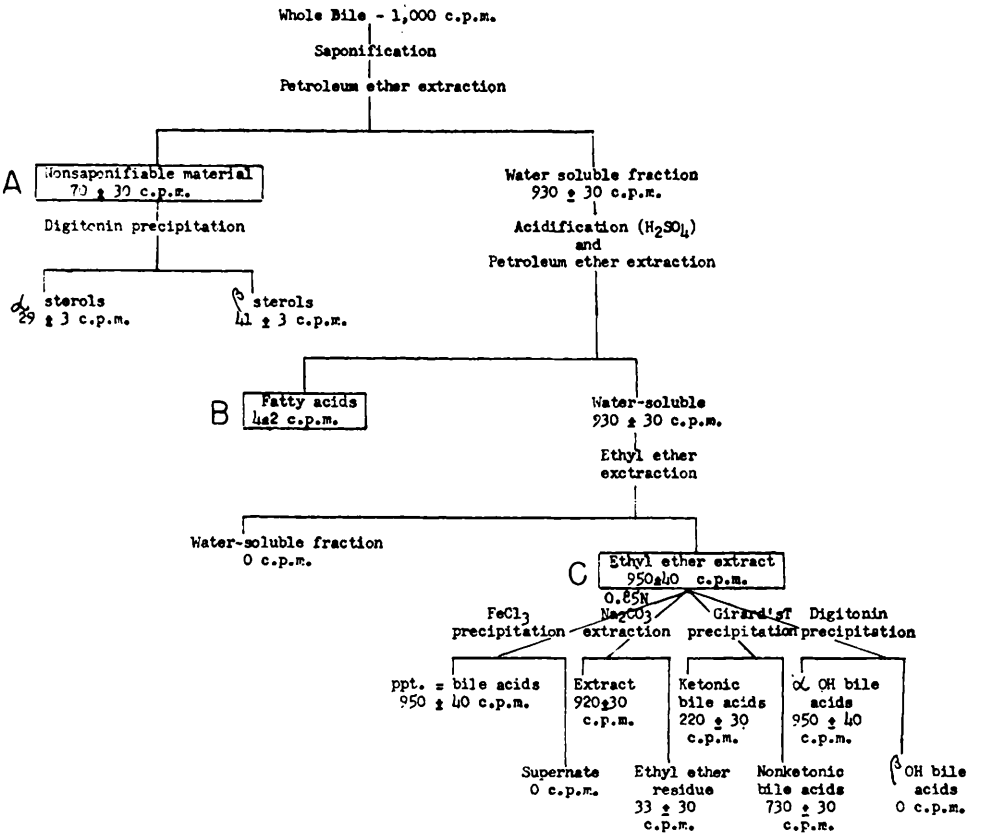

FIG. 1. Procedure for fractionation of bile. The C^{14} activity in the bile samples analysed has been assigned an arbitrary value of 1000 c.p.m. The actual total activity in the samples analysed was approximately 20000 c.p.m.

in saline with the aid of Tween 20(6) was injected into their tail veins. One group of rats treated in this manner was placed in metabolism cages for the collection of feces. A second group had cannulae placed in their common bile ducts as previously described(5); these rats were kept in restraining cages. The rats were raised on a diet low in cholesterol (about 10 mg per 100 g of diet) which was also fed during the experiment.

Procedures. Fractionation. Feces were collected for 15 days after the administration of the labeled cholesterol to normal rats. In the case of the bile duct-cannulated rats, bile was collected for 48 hours. The feces samples analyzed were obtained between 72 and 120 hours. The bile samples analysed were collected from 6 to 24 hours after the administration of the C^{14}. Flow sheets illustrating the fractionation procedures for bile and feces are shown in Fig. 1 and 2. *Preliminary extractions.* Feces collected for intervals of 24 or 48 hours were extracted 3 times with boiling ethyl alcohol, and the residue was transferred to a Soxhlet apparatus where it was extracted for 48 hours with ethyl alcohol. The extracts were combined and saponified as described below. It was noted that the alcohol removed all of the C^{14} activity from the feces. Samples of bile were not subjected to this preliminary extraction. *Saponification.* One ml of bile or of the alcoholic extract of feces was placed in a 125 ml Erlenmeyer flask, and 6 ml of 7N NaOH were added. A bubble stopper was placed in the flask which was then autoclaved for 3 hours at 15 lb pressure. This procedure was shown in preliminary experiments to saponify taurocholic acid completely. Heating on a steam bath for a similar length of time was inadequate for this purpose. *Extraction of nonsaponifiable material.* After saponification, an equal volume of 95%

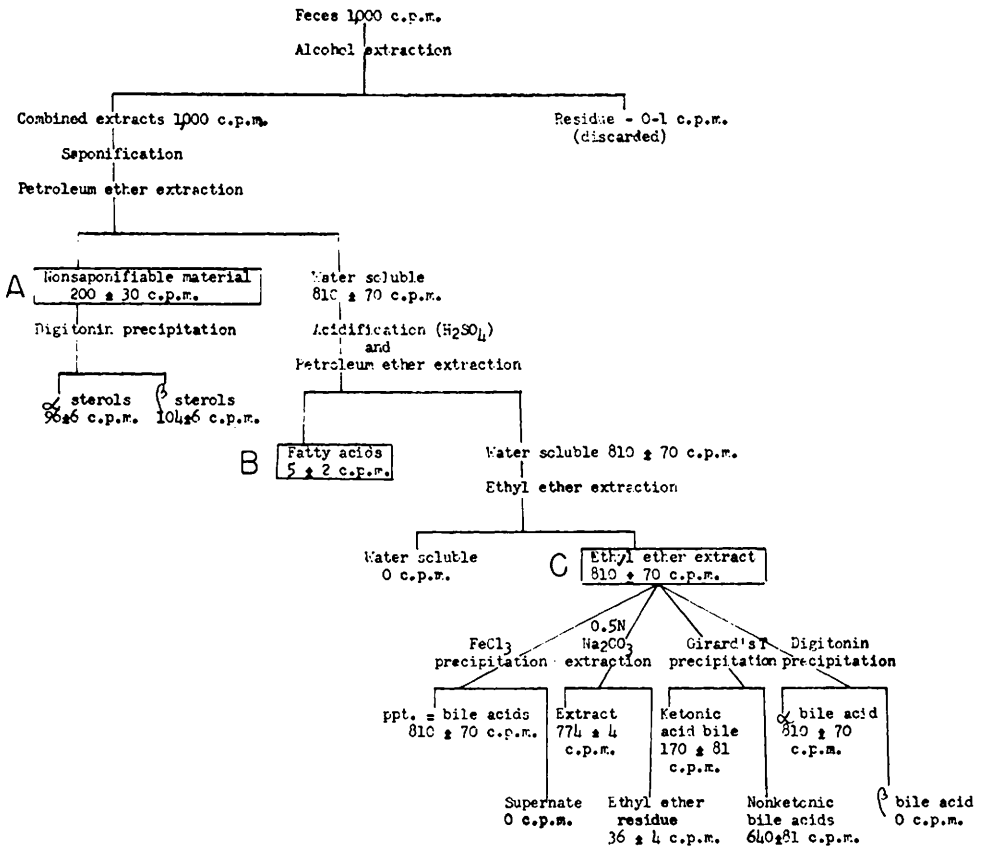

FIG. 2. Procedure for fractionation of feces. The C^{14} activity in the feces samples analysed has been assigned an arbitrary value of 1000 c.p.m. The actual total activity in the samples analysed was approximately 50000 c.p.m.

ethyl alcohol was added to the contents of the flask. An 8- to 10-fold excess of petroleum ether was next added, and the flask was vigorously shaken. The extraction with petroleum ether was repeated twice more. The extracts were then combined and concentrated on a steam bath, after which the concentrated material was transferred to a 25 ml volumetric flask. An aliquot of this nonsaponifiable Fraction A was mounted directly on an aluminum plate, and its C^{14} content determined in a flow gas counter. *Extraction of fatty acids.* The alcohol-water phase that remained after removal of the nonsaponifiable materials was then heated on a steam bath to remove the alcohol. The solution was acidified with H_2SO_4 to pH 1. Three petroleum ether extractions were carried out exactly as described for the nonsaponifiable material. These served to remove fatty acids; bile acids are insoluble in petroleum ether. *Extraction of bile acids.* The aqueous phase, which now contained neither fatty acids nor nonsaponifiable materials, was extracted 6 times with ethyl ether. This procedure removes bile acids. After 6 extractions, no C^{14} remained in the aqueous layer. The combined ethyl ether extracts were made basic with ammonium hydroxide, concentrated to a small volume, and transferred to a 25 cc volumetric flask with ethyl alcohol. An aliquot was then mounted on an aluminum plate and its C^{14} content determined. *Extractability of activity from ethyl ether extracts with 0.8 N Na_2CO_3.* An aliquot of the ethyl ether extract C was placed in a separatory funnel and extracted 3 times with equal volumes of 0.85 N Na_2CO_3. The ethyl ether was transferred to a volumetric flask and its C^{14} content determined.

Doubilet procedure for isolation of bile

acids. Another aliquot of the ethyl ether extract C containing the bile acids was taken to dryness on a steam bath. Fifty mg of cholic acid were added as carrier. The mixture was next taken up in dilute NaOH and transferred quantitatively to a 15 ml centrifuge tube. After the pH of the solution was brought to 6.8, the bile acids were precipitated as the iron salts exactly as described by Doubilet(7). The mixture was centrifuged at 2,800 r.p.m. for 15 minutes, the supernatant was decanted, and the precipitated bile acids were washed 3 times with 0.5 N Na_2SO_4. The combined supernatant and washings were evaporated to dryness, and the residue was exhaustively extracted with ethyl alcohol. The C^{14} activity of this alcoholic fraction which contained the nonbile acid activity was determined. *Ketonic bile acids.* A third aliquot of the ether extract C was used for the isolation of ketonic bile acids. They were isolated as the mercuric iodide salt of the hydrozone of the bile acids by the method of Hughes(8). The bile acids precipitated by this procedure were dissolved in pyridine, an aliquot of which was taken for C^{14} analysis.‡ *Digitonin-precipitable C^{14}.* A fourth aliquot of the ether extract C was taken to dryness, and the residue was transferred with ethyl alcohol to a 15 ml centrifuge tube. One mg of carrier cholesterol was added, and the Sperry-Webb procedure(9) for isolation of digitonin-precipitable materials followed. The washed precipitate was dissolved in methyl alcohol for determination of its C^{14} activity.

An aliquot of the nonsaponifiable Fraction A was also treated with digitonin exactly as described above.

Results. In the case of feces, we observed a difference between early and later samples in the ratios of nonsaponifiable C^{14} to total C^{14}. Some time is required to transport the C^{14}-containing bile to the excreted feces. Thus in the first 24-hour feces sample, nonsaponifiable C^{14} represented about 30% of the total C^{14}, whereas in feces samples collected at later intervals, the ratio fell to about 20% of total fecal-C^{14}. The higher value in the first 24-hour sample probably represents to a large extent material excreted directly through the intestinal wall, the major portion of which is nonsaponifiable. By the time the bile reaches the large intestine, the C^{14} it contributes to feces far outweighs that contributed by direct passage across the intestinal wall. Because of the above considerations, it would appear that later samples of feces present a truer picture of normal cholesterol excretion. Samples of feces collected between 72 and 120 hours after the administration of the labeled cholesterol were therefore chosen.

Bile. The distribution of the C^{14} activity is shown in Fig. 1. Little, if any, of the C^{14} activity was recovered in the fatty acid fraction.§ The nonsaponifiable fraction contained from 4 to 10% of the total C^{14} of the bile sample, slightly more than half of which was digitonin-precipitable (*i.e.*, presumably present as cholesterol, dihydrocholesterol, coprosterol). By far the major portion of the C^{14} was recovered in the ethyl ether extract of the acidified hydrolysate (Fraction C). The following evidence indicates that the C^{14} compounds in this fraction are bile acids: 1) insolubility in petroleum ether; 2) complete solubility in ethyl ether; 3) almost complete recovery in the acidic sterol fraction with the aid of 0.85 N Na_2CO_3; and 4) complete recovery of the activity in the bile acid fraction isolated by the Doubilet procedure. Since, in naturally occurring bile acids, the OH group at Carbon 3 is *trans,* none of the activity, as was to be expected, was precipitated with digitonin. About 30 per cent of the C^{14}-bile acids was found in the ketonic fraction.‡

Feces. The distribution of the C^{14} in various fractions (Fig. 2) resembled that observed for bile except that somewhat more of the activity was recovered in the nonsaponifiable fraction. The latter, of course, represents

‡ In the course of chromatographic separation of pure bile acids it has been observed that heating a neutral solution of sodium cholate will change its R_f. It is possible, therefore, that the isolation procedure used here could have altered the structure of the bile acids. Thus, the ketonic bile acids may possibly be artifacts.

§ It is of interest to note that after the administration of cholesterol-26-C^{14} to rats, practically none of the fecal C^{14} was recovered in the fatty acid fraction (Fraction B, Fig. 2) (unpublished observations).

the C^{14} contribution from the intestinal wall. Thus the principal C^{14} end product in feces as well as in bile is in the nature of a bile acid.

Discussion. Evidence is presented here to show that bile acids constitute the major excretion products of cholesterol in both bile and feces. This finding is not too surprising and, indeed, was strongly indicated by the previous demonstration that, following intravenous injection of cholesterol-4-C^{14}, bile constituted the chief route of excretion of the C^{14} into the intestine, and, further, that this carbon is not recoverable as a neutral sterol(5). The loss of carbons 26 and 27, but not of carbon 4, as CO_2 in expired air(10) likewise is in accord with the view that a bile acid is the excretion product for the nucleus of cholesterol. Finally, the demonstration of an enterohepatic circulation of a large portion of the cholesterol metabolic end product(11) agrees well with what has long been known concerning bile acid reabsorption and reexcretion into the intestine.

The results of these previous studies on cholesterol metabolism together with the evidence presented here on the nature of the cholesterol end products now make it possible to outline the major metabolic pathway of the cholesterol molecule in the body.

Ingested cholesterol is absorbed from the intestinal tract almost entirely by way of the lymph(12), a process in which bile plays an essential role(13). Following its absorption, about 80% of the terminal 2 carbons of the isooctyl chain is gradually oxidized and eliminated as CO_2 in the expired air(10). A number of tissues can oxidize the chain carbon but liver is most active in this respect, and this tissue probably accounts for the greatest amount of cholesterol destroyed in the body (6). Following the removal of the terminal carbons of cholesterol, and other less drastic alterations, the residue is excreted into the bile as bile acids. The remaining 15 to 25% of the cholesterol is eliminated either through bile or across the intestinal wall as neutral sterols containing 27 carbons.

A large portion of the bile acids excreted into the intestine is promptly reabsorbed into the body via the portal blood. These bile acids are then quickly reexcreted through the bile only to be reabsorbed a second time and again excreted. This enterohepatic circulation of carbon 4 of bile acids undoubtedly continues through many cycles but, with each cycle, a small amount of bile acid escapes to be eliminated in the feces. The total of these small increments of escaping bile acids finally represents between 75 and 85% of the fecal sterols. This, in addition to the 27-carbon containing sterols eliminated either directly through the intestinal wall or through the bile accounts for almost all of the cholesterol excreted from the body.

Summary. Cholesterol-4-C^{14} was administered intravenously to normal rats and the C^{14} end products in feces and bile were fractionated. 1. From 4 to 10% of the total C^{14} in bile and from 17 to 23% of fecal C^{14} were recovered in the nonsaponifiable fraction which includes cholesterol, dihydrocholesterol, coprosterol, etc. 2. Little if any of the C^{14} in either bile or feces was recovered in the fatty acid fraction. 3. Evidence is presented to show that about 80% of the fecal C^{14} and about 90% of the bile C^{14} are present in the form of bile acids. 4. The major metabolic pathways for the nucleus carbons of cholesterol are outlined.

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