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Occurrence of Lithocholic Acid in Feces of Healthy Men

Abstract. Crystalline lithocholic (3α-hydroxycholanic) acid was isolated from a pooled sample of feces from healthy men for the first time. This acid, which occurs in small amounts in human bile, was obtained by alcohol extraction, followed by solvent partition and chromatography. Under these conditions most of the acid was recovered in the form of its methyl ester.

Of the cholanic acids isolated from human bile, chenodeoxycholic (3α,7α-dihydroxycholanic) acid and cholic (3α,7α,12α-trihydroxycholanic) acid are the most abundant. Deoxycholic (3α,12α-dihydroxycholanic) acid was found in smaller amounts, but lithocholic (3α-hydroxycholanic) acid only rarely and in very small amounts (*1*). Little isolation work has been reported on the bile acids of human feces, but they are known to contain cholic (*2*) and deoxycholic acid (*3*). The occurrence of lithocholic acid in human feces has not yet been described. This bile acid was first found in gallstones of cattle by Hans Fischer in 1911 (*4*) incidental to his work on bilirubin. In the course of an extensive investigation of the lipid fraction of human feces (*5*), we have isolated lithocholic acid in pure form.

The isolation procedure involves a separation scheme similar to the one used by Dobriner *et al.* (*6*) for the isolation of steroids from urine. Fifty kilograms of fresh stool from about 20 healthy men was homogenized in ethanol containing 1 mole of hydrochloric acid per liter at 50°C for 7 hours, filtered, and concentrated in a vacuum. The concentrate was dissolved in 15-percent ethanol and extracted with chloroform. Acidic material was separated from the chloroform solution by extraction with 2*N* sodium hydroxide, and the neutral material was partitioned between 80-percent ethanol and petroleum ether. The material in the alcohol phase was separated into a ketonic and a nonketonic fraction by use of Girard's reagent, and the nonketonic fraction was separated into an alcoholic and a nonalcoholic fraction via the hemiphthalates.

The acidic fraction was subjected to a counter-current distribution in an *n*-heptane/97.5-percent aqueous acetic acid system (*7*), and a fraction of the aqueous phase was chromatographed on Celite, the solvent system of Matschiner *et al.* (*8*) being used. The fraction eluted with petroleum ether crystallized from aqueous ethanol and gave an infrared spectrum (*9*) which indicated the presence of lithocholic acid. Upon recrystallization from aqueous ethanol and again from aqueous acetic acid crystals of mp 176° to 181°C (*10*) [reported, 185° to 186° (*11*)] were obtained, which gave an infrared spectrum identical with that of authentic lithocholic acid. In another experiment the chromatographic fraction containing lithocholic acid was treated with diazomethane for conversion into the methyl ester. The latter was purified by chromatography on silica gel by the method of Wootton (*12*) and yielded a fraction which on recrystallization from aqueous methanol afforded crystals melting at 79° to 84°C (labile form). Methyl lithocholate, prepared, chromatographed, and crystallized as above, melted at 82° to 85°C [reported, 90° to 93°C (*13*)] and gave an infrared spectrum identical with that of the methyl ester of the isolated specimen. Alkaline hydrolysis of this material, followed by recrystallizations from aqueous ethanol and aqueous acetic acid, afforded crystals of mp 182° to 185°C. This substance showed no depression of the melting point upon admixture with authentic lithocholic acid and had the same infrared spectrum as the authentic acid.

The major portion of the lithocholic acid was isolated from the neutral fraction. When the neutral nonketonic alcohols were acetylated and chromatographed on alumina, by the method of Reichstein (*14*), the methyl ester of 3α-acetoxycholanic acid was obtained. The compound, rechromatographed and recrystallized from ether-petroleum ether, crystallized in oblong leaflets, mp 132° to 134°C [reported, 134° (*15*)]. The substance showed no depression of the melting point upon admixture of authentic methyl 3α-acetoxycholanate and gave the same infrared spectrum as the authentic ester. The analysis (*16*) showed C, 75.16; H, 10.47 (calcd. for $C_{27}H_{44}O_4$: C, 74.95; H, 10.25) and $[\alpha]_D^{20} + 48.6°$ in acetone (reported, $[\alpha]_D^{15} + 48.4 \pm 3°$ (*15*)). Saponification of this acetate with sodium methoxide gave the methyl ester of lithocholic acid, which crystallized from aqueous acetone in needles, mp 81° to 86°C (labile form) and showed no depression of the melting point upon admixture with authentic methyl lithocholate. The infrared spectrum of the product was identical with that of the authentic material and analysis showed C, 76.89; H, 10.68 (calcd. for $C_{25}H_{42}O_3$: C, 76.87; H, 10.84) and $[\alpha]_D^{20} + 30.9°$ in acetone (reported, $[\alpha]_D^{13} + 32.8 \pm 2°$ (*15*)).

While the methods employed do not permit an accurate quantitative determination of the amount of lithocholic acid present in human feces, it is estimated that they contain approximately 3 g/100 kg of wet weight, a concentration which is in the order of that in ox bile (*17*). Our methods give no indication of the form in which lithocholic acid is excreted, and further work will be required to account for the isolation of its methyl ester.

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Method for Determination of Oxygen-18 Content of Inorganic Phosphate

Abstract. The reaction of inorganic phosphate and mercuric cyanide at temperatures from 240° to 300°C leads to the quantitative conversion of the phosphate oxygens to carbon dioxide. This report describes the proper conditions for the advantageous use of this reaction for the determination of the oxygen isotope composition of the inorganic phosphate.

The mechanism of several enzymatic reactions has been successfully investigated through use of O^{18} as an isotopic tracer. Various phosphorylation reactions involving the transfer of oxygen between inorganic phosphate or phosphate derivatives and organic substrates have been particularly susceptible to analysis by this technique. These analyses invariably involve the determination of the O^{18} content of inorganic phosphate, and although the available methods for inorganic phosphate O^{18} determination give satisfactory results, they suffer from multiple disadvantages. The dehydration method (*1*), based on the pyrolysis of inorganic phosphate to H_2O and pyrophosphate, followed by equilibration of the H_2O with CO_2, is lengthy and time-consuming. Furthermore, since this method involves dilution of the phosphate oxygens, relatively large amounts of phosphate are needed for accurate determinations. A second general analytical method involves heating carbon and inorganic phosphate at 1350°C to yield CO (*2*). This method requires an elaborate apparatus and has the disadvantage that the product, CO, is not easily separable from air and has the same mass as N_2.

Table 1. Effect of varying amounts of KH_2PO_4 on the O^{18} concentration as measured in the CO_2.

Sample	Ratio mass 46/44	Atom-percent excess
1 mg $KH_2PO_4{}^{18}$*	0.026874	1.13
3 mg $KH_2PO_4{}^{18}$*	0.027445	1.15
5 mg $KH_2PO_4{}^{18}$*	0.027453	1.15
10 mg $KH_2PO_4{}^{18}$*	0.027229	1.15
50 mg $KH_2PO_4{}^{16}$	0.004072	0.0

* The theoretical O^{18} atom-percent excess was 1.2.

A procedure has been developed in this laboratory which largely circumvents these disadvantages (*3*). The method is similar to that developed by Rittenberg and Ponticorvo for the determination of O^{18} in organic compounds (*4*). The modified method involves heating KH_2PO_4 and $Hg(CN)_2$ (*5*) in a sealed tube for 1 hour at 250°C. Under these conditions, the phosphate oxygens are converted to CO_2 without dilution. The CO_2 is collected and subsequently introduced into the mass spectrometer (*6*). The original Rittenberg-Ponticorvo method, as developed for organic O^{18} determination, is not applicable to inorganic phosphate because of oxygen exchange reactions with the glass container (*7*).

The procedure is as follows:

1- to 50 mg samples of KH_2PO_4 are placed in break-seal tubes, as described by Rittenberg and Ponticorvo (*4*). The tubes are dried at 100°C under reduced pressures. Each break-seal tube is then thickened a short distance from the open end and cooled, and 25 mg of dry $Hg(CN)_2$ is introduced. The tube is evacuated to a final pressure of 7 to 10 μ-Hg, sealed, and heated at 250°C for 1 hour.

The CO_2 formed in the sealed tube is collected in the apparatus shown in Fig. 1. Following the introduction of the sealed tube and the assembly of the apparatus, the whole system is evacuated to a pressure of 7 to 10 μ-Hg without liquid N_2 over the second U-tube trap. Following evacuation, stopcock A_1 is closed, and the break seal is broken with the aid of the magnet. Stopcock C is then closed, trap 2 is immersed in liquid N_2, and stopcock A is opened to allow CO_2 to distill into trap 2. Any HCN which is formed is frozen out in the Dry-Ice acetone trap (trap 1). After 3 or 4 minutes, stopcocks B_1 and B_2 are closed and stopcock C is opened to evacuate any gas not frozen out in liquid N_2. Stopcock C is then closed, and trap 2 is removed from the apparatus and connected to the mass spectrometer.

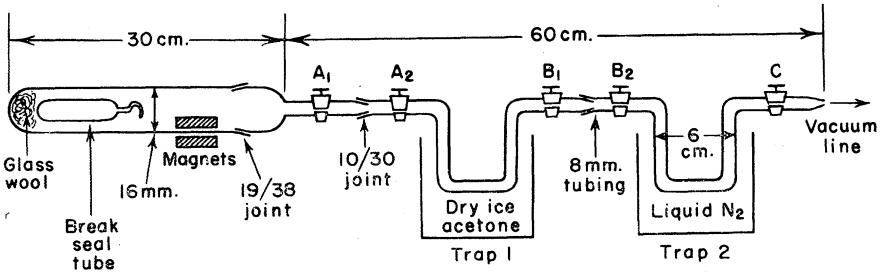

Fig. 1. Apparatus for collection of CO_2.

The results of a series of determinations are given in Table 1.

In a large number of determinations, the results were extremely consistent. The O^{18} concentration of KH_2PO_4 over a range of from 2 to 50 mg of the salt could be determined with a deviation of ± 0.5 percent. Furthermore, it would appear that the heating conditions do not have to be reproduced to any high degree of consistency. Over a temperature range of 230° and 300°C and a time range of 40 minutes to 2 hours, identical O^{18} values were obtained.

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Nuclear Sex of Patients with Testicular Tumors

Abstract. In view of the occurrence of tumors of the testis of "female" nuclear sex, the cells of the hosts of such tumors have been examined. The nuclear sex of a series of 75 such patients was found to be uniformly "male."

The introduction by Barr and Bertram (*1*) of a reliable technique for cytological determination of the chromosomal sex of an individual has had widespread repercussions in human pathology. One of the most interesting developments has been the discovery by Hunter and Lennox (*2*), amply confirmed by others (*3*) that teratomas in male patients may sometimes be of female nuclear sex, whereas "male" tumors are not seen in females. It is claimed that testicular tumors are much more common in malde-