SEPARATION OF THE ACID PRECURSORS OF CHOLESTEROL BY COLUMN CHROMATOGRAPHY*

by

LUCILE F. ADAMSON AND DAVID M. GREENBERG

Department of Physiological Chemistry, University of California School of Medicine, Berkeley, Calif. (U.S.A.)

The different acids that have been implicated as precursors of cholesterol can be separated by chromatography on a silica gel column. The methods now in use for the isolation of these acids are laborious and are restricted to individual members[1,2]. Small amounts of these acids added as carriers (*ca.* 5 μmoles) to denaturated tissue preparations can be recovered in good yield, thus making the procedure highly suitable for the quantitative study of the radioactive acid intermediates on the pathway to cholesterol from acetate or other ^{14}C-labeled substrates.

EXPERIMENTAL PROCEDURE

The procedure employed is a modification of the method of KINNORY, TAKEDA AND GREENBERG[3] for the separation of short chain mono and dicarboxylic acids. The column employed, the preparation of the silica gel for chromatography and the eluting solvents are the same as those described by KINNORY, TAKEDA AND GREENBERG[3]. Tissue proteins are removed by precipitation with 0.1 volume of 2 *M* perchloric acid instead of meta-phosphoric acid and the excess perchloric acid precipitated as the potassium salt.

In order to determine the completeness of tissue acid recovery, the carrier acids are added to the sample immediately after the addition of the perchloric acid rather than just before chromatography. After centrifugation, the protein precipitate is washed twice with small volumes of water (*ca.* 1 ml). The combined supernatants are neutralized to pH 7 by the addition of a few drops of 20% $KHCO_3$ and allowed to stand in a refrigerator. After removing the $KClO_4$ by centrifugation the supernatant solution is adjusted to pH 8 with dilute NaOH and evaporated to dryness in a heated vacuum desiccator. The residue is acidified and dissolved in a minimum amount of water. The salts precipitated by the addition of acetone-alcohol (1:2) are removed by centrifugation. The supernatant solution is again adjusted to pH 8 and evaporated to dryness. The residue is then acidified and placed on the column for chromatography.

RESULTS

With the benzene-ether solvent system of KINNORY, TAKEDA AND GREENBERG, separate titration peaks are obtained for β,β-dimethylacrylic acid (DMA), β-hydroxy-*iso*valeric acid (HIV), and β-hydroxy-β-methylglutaric acid (HMG). Acetic acid and β-methylglutaconic acid (BMG) are eluted together in another peak. All of the five acids are separated, however, if early in the fractionation a mixture of petroleum ether (boiling range 30–60°) and ethyl ether (1:1) is used in the separatory funnel in place of ethyl ether. This mixture serves to broaden the elution bands. Just before

* Aided by research grants from the Life Insurance Medical Research Foundation.

References p. 518.

or immediately after the acetic acid is eluted, the above mixture must be replaced with 100% ethyl ether in order to assure the early elution of the β-hydroxy-β-methylglutaric acid. The *cis* and *trans* isomers of β-methylglutaconic acid are also separated by this procedure. The chromatogram showing the titration data on a known mixture of the five acids separated with this system is given in Fig. 1.

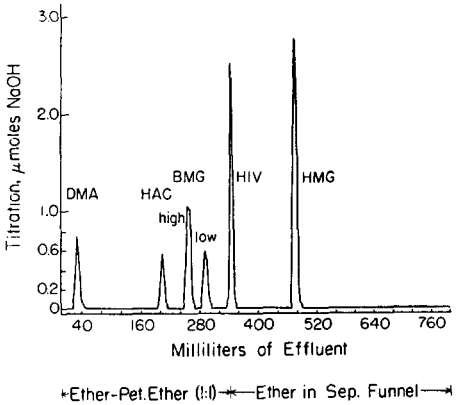

Fig. 1. Chromatography of mixture of known acids added directly to silica gel column. DMA, β-β-dimethylacrylic acid; HAc, acetic acid; BMG, β-methyl glutaconic acid; HIV, β-hydroxy*iso*valeric acid; HMG, β-hydroxy-β-methylglutaric acid.

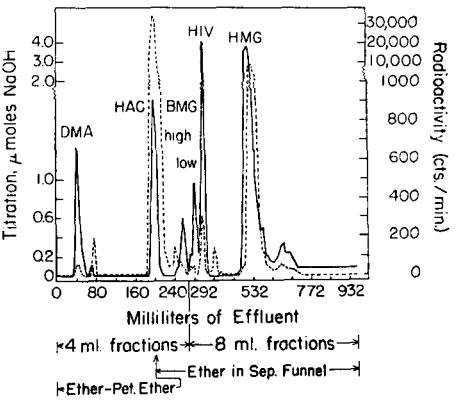

Fig. 2. Chromatogram of incubation of $CH_3{}^{14}COONa$ with liver slices. Recovery of acids added to incubation mixture. Solid line, titration; dotted line, radioactivity.

Fig. 2 shows the titration and radioactivity chromatograms of a sample prepared from a homogenate of rat liver slices (1.5 g), which had been incubated with $CH_3{}^{14}COONa$ in 4 ml of potassium phosphate buffer (pH 7) containing niacin amide (0.03 *M*) and $MgCl_2$ (0.004 *M*). The figure shows that the radioactivity peaks correspond to the titration peaks of DMA, HAc, HIV, and HMG. Radioactivity is usually observed in DMA, although it is low in this instance. The isolated BMG is often radioactive, although not in the sample illustrated here. The activity of HMG is generally remarkably high, sometimes approaching that of the recovered acetate.

Recoveries of carrier acids for a sample experiment are shown in Table I. The

TABLE I

RECOVERY OF ACID PRECURSORS OF CHOLESTEROL IN PURE SOLUTION AND WHEN ADDED TO A TISSUE HOMOGENATE

Carrier acid	*A*		*B*	
	Amount added μ equiv.	*Recovered %*	*Amount added μ equiv.*	*Recovered %*
DMA	4.87	104	4.88	86
HAc	0.83	98	1.90	174
BMG	4.08	96	5.08	63
HIV	4.98	94	3.98	88
HMG	5.48	101	6.12	250

A. Carriers added directly to column.
B. Carriers added to homogenate and reisolated.

References p. 518.

recoveries observed when a known acid mixture is applied directly to the column are also shown. Of the acids discussed here, only HAc and HMG were present in the homogenate sample in amounts great enough to be detectable by titration without the addition of carrier acids. The 250% recovery shown for HMG reflects the presence of relatively large amounts of this acid in liver. Endogenous acetate and substrate acetate result in high acetate recovery. Recoveries of BMG are often low and its destruction during isolation may also contribute to the acetate peak.

In conjunction with the silica gel chromatography of the water soluble acids, the non-saponifiable fraction of an aliquot of the same incubation mixture can be chromatographed on alumina to isolate ^{14}C-labeled cholesterol and squalene. The method is being used to study the synthesis of cholesterol precursors under various conditions and in various tissue systems.

SUMMARY

A chromatographic procedure using a silica gel column is described for the separation of the proposed acid precursors of cholesterol; namely, β,β-dimethylacrylic acid, β-hydroxy*iso*valeric acid, β-hydroxy-β-methylglutaric acid, β-methylglutaconic acid, and acetic acid. The method is very useful for the isotopic tracer study of the biosynthesis of cholesterol.

RÉSUMÉ

Les auteurs décrivent une méthode de chromatographie sur colonne de gel de silice permettant de séparer les précurseurs acides supposés du cholestérol, à savoir, l'acide β,β-diméthylacrylique, l'acide β-hydroxy*iso*valérique, l'acide β-hydroxy-β-méthylglutarique, l'acide β-méthylglutaconique et l'acide acétique. La méthode rend de grands services dans l'étude de la biosynthèse du cholestérol au moyen de marqueurs isotopiques.

ZUSAMMENFASSUNG

Eine chromatographische Methode für die Trennung der vorgeschlagenen sauren Vorgänger von Cholesterol, an Hand einer Silikagelsäule, wird beschrieben; und zwar handelt es sich dabei um β-β-Dimethylakrylsäure, β-Hydroxy*iso*valeriansäure, β-OH-β-Methylglutarsäure, β-Methylglutakonsäure und Essigsäure. Diese Methode ist von grossem Nutzen für das Studium der Biosynthese von Cholesterol an Hand von isotopischen Markiersubstanzen.

REFERENCES

1 J. L. RABINOWITZ, *J. Am. Chem. Soc.*, 77 (1955) 1295.
2 H. RUDNEY, *J. Am. Chem. Soc.*, 77 (1955) 1698.
3 D. S. KINNORY, Y. TAKEDA AND D. M. GREENBERG, *J. Biol. Chem.*, 212 (1955) 379, 385.

Received May 8th, 1955

Note added in proof

Destruction of BMG is decreased if the protein is precipitated by immersion in boiling water rather than by perchloric acid.