Characterization of Rat Tissues by Gas-Liquid Chromatography with the Electron-Capture Detector* (34042)

MICHA THEINER[1] AND LEO FRIEDMAN

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

We examined the feasibility of obtaining gas-liquid chromatographic (GLC) profiles of the distribution of electron-capturing (EC) components in mammalian tissues. The GLC profiles of the unsaponifiable lipids (UL) of rat tissues, monitored simultaneously by a flame ionization detector (FID) and an electron capture detector (ECD), were characteristic of both the source tissue and the individual rat. The GLC profiles of over 100 samples were only superficially similar to each other. Differences in the relative amounts of the EC components created individually characteristic GLC patterns, which were magnified by the removal of cholesterol from the lipid samples. This method provides a means of focusing on the EC lipids, which constitute only several parts per million in the tissue and have molecular weights of several hundred atomic mass units. Some of the EC components observed in the GLC patterns may be the products of pyrolysis of retinol and related compounds.

This study was prompted by theoretical (1, 2) and experimental (3, 4) demonstrations of the importance of EC compounds in biology, the development of the ECD by Lovelock (5), and the application of the ECD to compounds of biological importance (6–8). The ECD is a very sensitive and selective GLC ionization detector. It is very sensitive to highly halogenated compounds, such as the chlorinated pesticides, and thus has become a standard tool for the detection and estimation of pesticides in trace amounts (in the picogram or parts-per-billion range) (9).

At the same time there have been a number of reports that biological samples can be characterized by the GLC patterns or "fingerprints" of their extracts (10–13). Coupling of the selectivity of the ECD response with the individuality of the GLC profiles enhanced the instrumental characterization of biological samples (14–19). These reports involved the study of highly volatile compounds in bacteria, plants, and airborne odors. We have extended the scope of these studies to extracts of mammalian tissues and the larger lipid molecules they contain.

Materials and Methods. We performed all GLC analyses in a F & M model 400 "Biomedical" gas chromatograph (Hewlett-Packard Corp., F & M Division, Avondale, Pa.), equipped with a stream-splitter, FID, ECD, and linear temperature programming. All surfaces which came in contact with the sample, except the detectors, were glass. The GLC column was made of 4.0 g (105 cm) packing in a U-shaped glass tube 0.35 cm in diameter. The packing was 0.4% SE-30 silicone gum (General Electric Corp., Camden, N. J.) on acid-and-base-washed and silanized Chromosorb-W, 60-80 mesh (Analab Inc., Hamden, Conn.), prepared by the solution-coating method (20). Lipid samples were routinely prepared in "Spectrograde" isooctane (Fisher Scientific Co., Pittsburgh, Pa.) as a 10 mg/ml solution and 3.0 μl of this solution applied by on-column injection. The samples were flash-vaporized onto the column at 190–210° and carried through the column by a 60 ml/min stream of helium. All gases were obtained from The Matheson Co., East Rutherford, N. J. The column temperature was maintained at 133° for 9 min, increased at a rate of 30°/min to 200°, and maintained at this temperature for 20 min. The eluate

* Supported by NIH Grant ES-00183; reported in abstract form in Federation Proc. 26, 852 (1967). Contribution No. 1415 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

[1] Present address: Department of Biochemistry, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pa., 15213.

was divided by the stream-splitter into two equal portions. One was mixed with hydrogen (40 ml/min) and examined in a FID, which was supplied with 300 ml/min of air. The flame temperature was quite constant throughout and was maintained below 400° for enhanced sensitivity. The other half of the column eluate was mixed with 90 ml/min of "P-10" proportional counting gas; it then entered the ECD, which was maintained at a constant temperature of 210°. The ECD was the original one supplied with the F & M model 400 and operated with a pulse-sampling of 0.75 μsec duration every 150 μsec. The signal from each detector was amplified by a separate electrometer and recorded by a separate ElekroniK-18 recorder (Minneapolis-Honeywell, Philadelphia, Pa.). The records were superimposed for comparisons.

We saponified fresh whole organs from normal adult male rats (200–400 g) by heating them in methanol containing 50% aqueous potassium hydroxide (4 ml/g of tissue) under reflux for 90 min. After cooling and diluting them with an equal volume of water we extracted them with "Nanograde" hexane (Mallinckrodt Chemicals, St. Louis, Mo.). We washed the extract with dilute potassium hydroxide and with water saturated with sodium chloride. We then dried it under a stream of high purity nitrogen that had just passed over activated "Molecular Sieve" 13-X pellets (Analab Inc., Hamden, Conn.). The solutes in this extract are the so-called unsaponifiable lipids (UL). In a group of six rats we obtained (mg/g of wet wt; mean ±SD): 13.3 ± 6.1 from the brains, 4.8 ± 1.1 from the kidneys, 3.3 ± 0.6 from the livers, and 2.4 ± 2.7 from the hearts. A typical GLC profile of a sample of rat liver UL is shown in Fig. 1. The major peak of the UL is cholesterol; the second identifiable component is squalene, which appears in the GLC elution pattern just before cholesterol. We ascertained the identity of these peaks by chromatography with added internal standards on both a nonpolar liquid (SE-30) and a polar one (Carbowax-20M). Neither cholesterol nor squalene gives a positive ECD response. Squalene can serve as an internal marker for both the retention time and FID response of the EC components. To focus on the latter, we removed the cholesterol by precipitation as the digitonide (21).

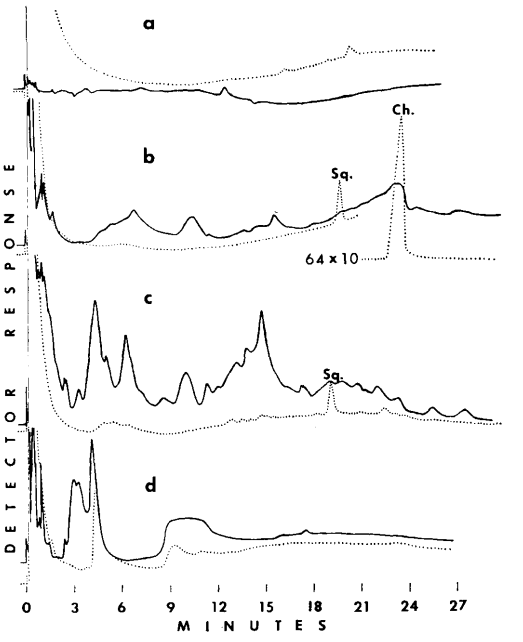

FIG. 1. Programmed temperature GLC patterns of: (a), solvent blank of the procedure; (b), total UL (30 μg from 10.7 mg of fresh rat liver); (c), the same UL (30 μg from 71 mg of liver) after removal of cholesterol by precipitation with digitonin; (d), the saponifiable (acid) lipids (30 μg from 3.8 mg of the same liver). GLC conditions were described in the text. (. . . .), FID, 350–390°, attenuation 64 × 1; (——), ECD, 210°, attenuation 128 × 10; Ch. = cholesterol; Sq. = squalene.

Results and Discussion. Figure 1 shows a typical GLC pattern of the UL remaining in solution (in 50% aqueous ethanol) after treatment with digitonin. The cholesterol peak is absent and the peaks of EC components are enlarged. A 4- to 20-fold concentration was achieved, since about 85 ± 10% of the UL were precipitated by the digitonin. The aqueous solvent required for this procedure may precipitate some UL other than cholesterol, depending upon their individual concentrations and solubility properties. In some cases, such as the one illustrated in Fig. 1, the proportion of squalene in 30

μg of UL did not increase after the removal of cholesterol. Another difficulty, which limits the use of this selective precipitation, is that the digitonin may add a considerable number of EC components to the sample. In fact, we found that commercial digitonin contains a large number of EC compounds. We therefore removed them by exhaustive extraction with hexane until aliquots of the extracts gave no significant EC peaks when analyzed by the GLC procedure.

We obtained the acid or "saponifiable" lipids by acidification of the alkaline digest, from which the UL had been removed, and extraction with diethyl ether. A typical GLC pattern of the acid lipids is shown in Fig. 1. Since the number of different EC components is apparently not very large and the pattern not as interesting as that of the UL, we did not study this fraction.

Figure 2 shows the GLC patterns of UL that had been treated with digitonin. The UL were from the grain, liver, heart, and kidneys of the same rat. Most of the EC components can be seen, in different amounts, in all the patterns. Some of the peaks seem to be present only in certain samples. Particularly outstanding are the brain UL. Even when cholesterol was not completely removed, the ECD response to the remaining UL gave a pattern that is distinctly different from those of the other tissues. On the other hand, the GLC patterns of heart and kidney UL are most similar. We found that SE-30 gave the best definition of peaks in these GLC patterns.

Some of the EC peaks may represent preexisting cellular lipids. Some may represent artifacts of the isolation procedure, *i.e.*, derivatives or breakdown products of the cellular lipids. Still others may represent the products of pyrolysis of the UL in the GLC flash vaporizer and column. The most likely EC lipids may be the retinenes (vitamin A), tocopherols (vitamin E), calciferols (vitamin D), and some 17-ketosteroids (15, 18). Most of these survive the high temperature of GLC without any apparent pyrolysis. The members of the vitamin A group are apparently pyrolyzed in the GLC and yield characteristic patterns of pyrolysis products. For example, retinol yields patterns like the one shown in Fig. 3. This pattern is similar to the

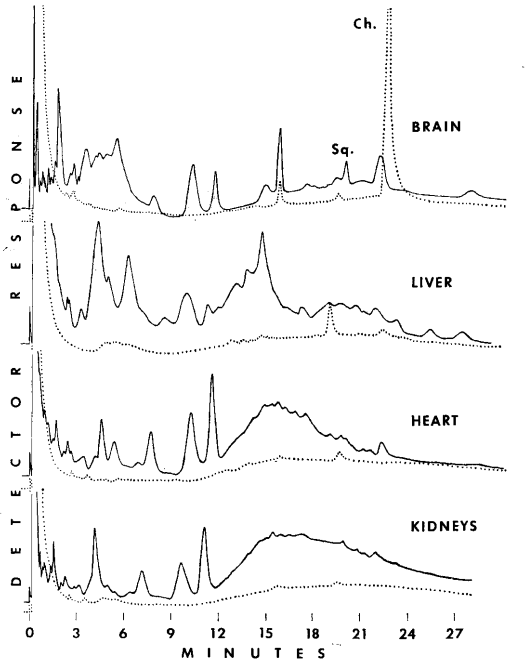

Fig. 2. GLC patterns of UL of different tissues from the same rat after the removal of cholesterol (Ch.) by precipitation with digitonin: brain, 61 mg; liver, 71 mg (the same extract as in Fig. 1); heart, 18 mg, and kidneys, 19 mg. GLC conditions were as described in the text and in Fig. 1.

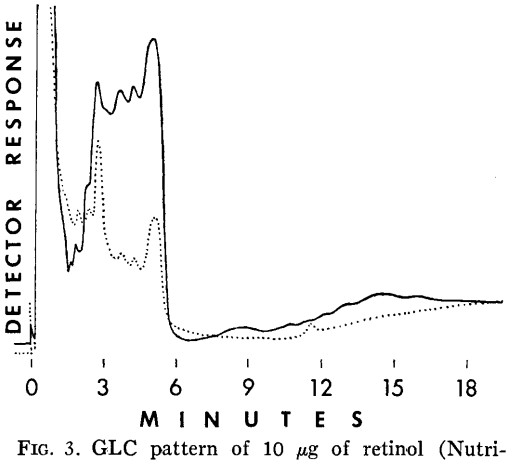

Fig. 3. GLC pattern of 10 μg of retinol (Nutritional Biochemicals Corp., Cleveland, Ohio). GLC conditions: 140° for 9 min, increased at 30° min to 190°. (....), FID, attenuation 16 × 1; (—) ECD, attentuation 128 × 10.

first few peaks of the liver UL shown in Figs. 1 and 2. Since the identity of the peaks is not known and the response of the ECD to various EC compounds varies over an extremely wide range (6–8), it is not possible to estimate the quantities of the EC components. From the response of the FID and experience with some pure compounds, it is obvious that the EC components constitute less than 0.1% of the UL and are therefore on the order of a few micrograms per gram of fresh tissue.

Summary. The unsaponifiable lipids (UL) of rat tissues include a number of components which possess moderate affinities for electrons with thermal energies. These can be separated and analyzed by gas-liquid chromatography coupled with simultaneous detection by flame ionization and electron capture. Different rat tissues yield characteristic chromatographic patterns.

1. Szent-Györgyi, A., "An Introduction to Submolecular Biology." Academic Press, New York (1961).

2. Pullman, B. and Pullman, A., "Quantum Biochemistry." Wiley (Interscience) New York (1963).

3. Szent-Györgyi, A., Isenberg, I., and Baird, S. L., Jr., Proc. Natl. Acad. Sci. U. S. **46**, 1444 (1960).

4. Epstein, S. S., Bulon, I., Koplan, J., Small, M., and Mantel, N., Nature **204**, 750 (1964).

5. Lovelock, J. E., Anal. Chem. **35**, 474 (1963).

6. Lovelock, J. E., Anal. Chem. **33**, 189, 729 (1961).

7. Zlatkis, A. and Lovelock, J. E., Clin. Chem. **11**, 259 (1965).

8. Simmonds, P. G., Ph.D. thesis, University of Houston (1966) (University Microfilms, Inc., Order No. 66-15, 279).

9. Burke, J. and Giuffrida, L., J. Assoc. Offic. Agr. Chem. **47**, 326 (1964).

10. Abel, K., DeSchmertzing, H., and Peterson, J. I., J. Bacteriol. **85**, 1039 (1963).

11. Reiner, E., Nature **206**, 1272 (1965).

12. O'Brien, R. T., Food Technol. **21**, 1130 (1967).

13. MacGee, J., J. Gas Chromatog. **6**, 48 (1968).

14. Oaks, D. M., Hartman, H., and Dimick, K. P., Anal. Chem. **36**, 1560 (1964).

15. Dugan, P. and Lundgren, D., Anal. Biochem. **8**, 312 (1964).

16. Henis, Y., Gould, J. R., and Alexander, M., Appl. Microbiol. **14**, 513 (1966).

17. Dravnieks, A. and Krotoszynski, B. K., J. Gas Chromatog. **4**, 367 (1966).

18. Simmonds, P. G., Pettitt, B. C., and Zlatkis, A., Anal. Chem. **39**, 163 (1967).

19. Mitruka, B. M., Alexander, M., and Carmichael, L. E., Science **160**, 309 (1968).

20. Wells, W. W., Sweeley, C. C., and Bentley, R., *in* "Biomedical Applications of Gas Chromatography" (H. Szymanski, ed.), p. 201. Plenum Press, New York (1964).

21. Windaus, A., Chem. Ber. **42**, 238 (1909).

Received Feb. 5, 1969. P.S.E.B.M., 1969, Vol. 131.