TWO-DIMENSIONAL PAPER CHROMATOGRAPHIC SYSTEMS WITH HIGH RESOLVING POWER FOR AMINO ACIDS

by

ROBERT R. REDFIELD

Section on Cellular Physiology, National Heart Institute, Bethesda, Md. (U.S.A.)

A system is described in this note which makes possible the separation of all the amino acids occurring in protein hydrolysates on a single small chromatogram.

Schleicher & Schuell No. 507 was found to be the preferable filter paper. The chromatograms obtained with this paper were free of "beards", and the spots were more even in outline. Squares 13¾ cm × 14½ cm were used. (This represents $^1/_{16}$ of the large sheets 55 cm × 58 cm provided by the manufacturer.) The spot containing the amino acid mixture was placed 2 cm from each margin of the paper, preferably in a circle less than 0.5 cm in diameter (1 μl or less), and neutralized over an atmosphere of ammonia vapour. Squares twice this size in each dimension can be used if a larger application spot is used, or if one prefers a wider scattering of the amino acid spots.

An apparatus such as that described by DATTA *et al.*[1] would appear to be ideal for developing these chromatograms, although we used beakers and museum jars for ascending chromatography.

Solvents: The solvents used were not redistilled or otherwise purified. Proportions given below are volume for volume.

In the first dimension, a mixture of methanol-water-pyridine (80/20/4) was used. The time of development for the 14 cm squares in small ascending chambers was 2–3 hours. For the larger 28 cm squares in larger chambers, 8–9 hours was required. The chromatograms were then removed and allowed to dry at room temperature for about 15 minutes.

In the second dimension, a modification of one of the solvents described by BOISSONNAS[2] was used: *tert.*-butanol-methyl ethyl ketone-water-diethyl amine (40/40/20/4). Time of development was 2½–5 hours for the smaller squares, or 12–16 hours for the larger squares.

An exact tracing of a small chromatogram developed with this solvent system is given in Fig. 1. A number of other amino acids found in biologic fluids but not in acid protein hydrolysates were included in the mixture applied and are noted in dotted outline.

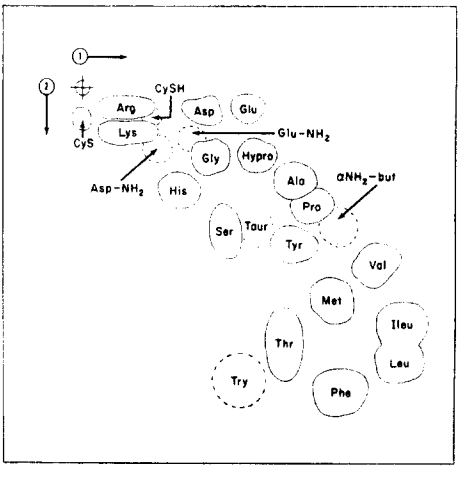

Fig. 1. Small chromatogram reduced to ½ actual size. The synthetic mixture contained 0.01 μM of each amino acid. Solvent 1: methanol-water-pyridine (80/20/4). Solvent 2: *tert.*-butanol-methyl ethyl ketone-water-diethyl amine (40/40/20/4). Run at room temperature of 22 to 25° C. Abbreviations as used by BRAND AND EDSALL[4].

An alternative system which provided a wider scattering of the amino acids occupying the upper left-hand corner, but which provided no separation for leucine-*iso*leucine or threonine-tryptophan was as follows:

For the first dimension, a mixture of methanol-water-pyridine (60/20/20) was used. Ascending time for the 28 cm squares was 8–14 hours.

The second dimension was run using a mixture of *n*-propanol-water-diethylamine (85/15/2 to 4). The development time was 12–18 hours.

On these chromatograms cysteine was completely separated from arginine or lysine. Ornithine, when present, occupied a position between arginine and lysine and nearer lysine.

Before the chromatograms were treated with ninhydrin, it was imperative that they hang free in an atmosphere of steam (furnished in the present case by an autoclave with the exhaust open) for five to eight minutes to insure removal of the adsorbed diethyl amine. Without such steaming the papers were too basic for good colour development. A near optimal spraying and heating technique has been described earlier[3].

The samples must be essentially free from salt in order to obtain well defined patterns as shown in Fig. 1. In the present experiments, desalting was carried out on a short column of a strong sulfonate

resin (such as Nalcite HCR) in the acid cycle followed by washing with water to remove anions, and displacement of the amino acids with 1 *N* ammonium hydroxide.

Sensitivity was increased by virtue of the smaller spots obtained with smaller chromatograms. Quantities of 0.005 μM gave good dark spots.

REFERENCES

1 S. P. DATTA, C. E. DENT AND H. HARRIS, *Science*, 112 (1950) 621.
2 R. A. BOISSONNAS, *Helv. Chim. Acta*, 33 (1950) 1966.
3 R. R. REDFIELD AND E. S. G. BARRON, *Arch. Biochem. and Biophys.*, 35 (1952) 443.
4 E. BRAND AND J. T. EDSALL, *Ann. Rev. Biochem.*, 16 (1947) 224.

Received January 7th, 1953

THE BIOSYNTHESIS OF RADIOACTIVE CHOLESTEROL BY PARTICLE-FREE EXTRACTS OF RAT LIVER*

by

JOSEPH L. RABINOWITZ AND SAMUEL GURIN

Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia (U.S.A.)

The biosynthesis of cholesterol has hitherto been accomplished *in vitro* by the use of tissue slices[1,2,3] or homogenates[4,5]. It is the purpose of this communication to report on the successful fractionation of rat liver homogenate, and the preparation from it of a water-soluble enzyme system capable of incorporating ^{14}C-labelled acetate into cholesterol.

Rat livers (2 g) were rapidly homogenized for 15–20 sec at 0° C in a loose-fitting Potter-Elvehjem glass homogenizer with two volumes of buffer (K_2HPO_4 0.019 *M*, KH_2PO_4 0.005 *M*, $MgCl_2$ 0.007 *M*, nicotinamide 0.03 *M*, sucrose 0.18 *M* pH 7.6). The homogenate was centrifuged at 3000 r.p.m. for 10 minutes (600 × *g*) to remove cell debris, cells, and nuclei. A mitochondrial fraction, which was obtained from the supernatant homogenate by centrifugation at 20,000 r.p.m. for 40 minutes (33,000 × *g*), was washed with cold buffer solution and resedimented. The supernatant fraction was centrifuged at 37,000 r.p.m. for 30 minutes (100,000 × *g*) to remove any remaining particles. The packed mitochondria were lysed by the addition of an equal volume of cold water, followed by occasional and gentle stirring for one hour at 0° C. The complete water-soluble enzyme system was prepared by the addition of clear supernatant fluid (4–5 volumes) to the lysed suspension followed by centrifugation at 37,000 r.p.m. for 30 minutes.

After incubation, the cholesterol in each sample was recovered as the digitonide[3] and plated. In some instances the cholesterol-digitonide obtained from the water-soluble enzyme system was converted into cholesterol dibromide and assayed again for radioactivity, with essentially no change.

Washed mitochondria plus supernatant fluid also provides a system which appears to incorporate acetate into cholesterol as efficiently as does the water-soluble enzyme system. Further details will be reported in a later communication.