動物組織のクエン酸回路の酸の高速液体クロマトグラフィーによる分離

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Separation of Citric Acid Cycle Acids from Animal Tissues by High-Performance Liquid Chromatography

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ABSTRACT. A method for separation of the acids of the citric acid cycle and related pathways was developed. The acids were separated by high-performance anion-exchange liquid chromatography on a short column of a strong anion-exchange resin (IEX 520AE) which was eluted with 1/30 M potassium phosphate buffer containing 27.5% (v/v) acetonitrile (pH 3.0) at 40°C. Compounds in the column effluent were detected by ultraviolet spectrophotometry at 210nm. This method was used in the analysis of acids of the citric acid cycle of rat brain and liver and of products of the reaction of succinic thiokinase or fumarase with extracts of animal tissues.—KEY WORDS: anion exchange, citric acid cycle liquid chromatography, UV.

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The citric acid cycle is the set of aerobic reactions responsbile for the energy-yielding oxidative degradation of pyruvate to carbon dioxide. In nearly all organisms it serves as the central pathway that integrates the metabolic flow of carbon, in conjunction with the process of oxidative phosphorylation, and as the major source of metabolic energy in the form of ATP. The enzymes and organic acids of the citric acid cycle have been extensively studied [3, 12] by many of possible methods for the separation [1, 8, 11, 20]. These methods involue liquid, paper, or gas chromatographic determination, or enzymatic analysis. The acids of the citric acid cycle are not stable, so time-consuming or intricate extraction procedures, lower the detection sensitivity. A repid and simple method is desirable.

Turkelson and Richards [17] have reported that the acids of the citric acid cycle can be separated on a column of a strong cation-exchange resin (Aminex 50W-X4) and detected by ultraviolet (UV) spectrophotomery at 210nm. This liquid chromatographic analysis is superior to the other methods heretofore reported, but is difficult to be applied to samples from animal tissue, since such samples are usually contaminated with various amino acids, peptides, and uncleic acids, all of which absorb UV region.

Here, a liquid chromatographic procedure was described that separated lactate, pyruvate, and the acids of the citric acid cycle from animal tissues within 30 min. The method is based on ion exclusion and partition chromatography with continuous UV monitoring of the column effluent at 210nm. The acids are separated on a column of strongly basic anion-exchange resin. A preliminary report appeared in the 1982 Annual Meeting of the Japanese Society of Veterinary Science.

MATERIALS AND METHODS

High-performance liquid chromatography: The experiments were performed with a JASCO trirotor III (Tokyo, Japan). The apparatus was equipped with an oven with a thermostat and a hand-injector. The column

effluent was monitored at 210 nm with a UV detector (Uvidec 100-III, JASCO). The flow cell volume was 8 μl. The analytical column (7.6×100 mm) was packed with 5-μm IEX 520QAE (Toyo Soda Mfg. Co., Ltd., Tokyo, Japan) with a mixing packer (Gasukuro Kogyo Inc., Tokyo, Japan). The packing solvent was methanol. The primary eluent was 1/30 M potassium phosphate buffer with 27.5% (v/V) acetonitrile, with a final pH of 3.0, at 40°C. Before use, each solution was filtered through a membrane filter (0.45-μm pores, Sartorius Co. and Sumitomo Electric Co., Ltd., Tokyo, Japan) and degassed with a vacuum pump.

Sample preparation: Male Wistar rats (250–300 g body weight) were used. After decapitation, about 2 g wet weight of the brain and the liver was deproteinized by homogenization into 9 vol of 10% cold perchloric acid. The homogenate was centrifuged at 25,000×g for 20 min, and the supernatant was adjusted to pH 7.0 with potassium hydroxide. After the precipitated potassium perchloric acid was removed by centrifugation at 25,000×g for 20 min, the supernatant was adjusted to pH 2.0 with 1 N HCl. The acidic solution was layered on the top of a column (1.0×5.0 cm) containing Dowex 50W-X8 (200–400 mesh) and eluted with 3 vol of 0.01 N HCl. Before the solution was layered on the column, the resin was equilibrated to pH 2.0 with 0.01 N HCl. The effluent was condensed with a vapor mixture, adjusted to 500 μl with distilled water, and injected into the analytical column in a volume of 10 μl.

For the analysis of enzyme reactions, 50 μl of the final biological extract was diluted with 450 μl of distilled water and adjusted to pH 7.4 with 2 N NaOH. Two units of succinic thiokinase or 1 unit of fumarase was added to this solution. As for succnic thiokinase, 1 mg of both GTP and CoA were added to the dilute solution before pH was adjusted. Incubation was carried out at 40°C for 120 min. Then an aliquot (25 μl) was injected directly into the analytical column at 30-min intervals from the start of incubation.

Chemicals: Fumarase, succinic thiokinase, GTP, CoA, lactate, succinyl CoA, and oxaloacetate were obtained from Sigma Chemical Co. (St. Louis, U.S.A.), acetic acid, fumarate, malate, α-ketoglutarate, citrate, and isocitrate, from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and succinate and pyruvate, from Kantoh Chemicals Co. (Tokyo, Japan). Since a special grade acetonitrile reagent still contained many impurities, the acetonitrile used was purified by the method of Walter and Ramley [18].

RESULTS

Fig. 1 demonstrates the effects of pH, ionic strength, and acetonitrile concentration of the eluent on the separation of some organic acids. Changes in the column temperature caused little change in the retention time (data not shown). When the eluent pH was increased to 3.8, the retention times of pyruvate and α-ketoglutarate were markedly lengthened, and the peaks of citrate and pyruvate partly overlapped. Absorption phenomena which sometimes occurs in ion-exchange chromatography are often induced by the use of distilled water or eluents of low ionic strength. The middle panel of Fig. 1 shows the effects of the ionic strength of the eluent on the separation of some organic acids. When the organic acids were eluted by 1/50 M potassium phosphate buffer, the retention times were longer and the peaks were distorted. With 1/20 M potassium phosphate buffer, the acids were eluted more rapidly than with 1/50 M potassium phosphate buffer, but the peaks congregated. An absorption effect was not found with 1/30 M potassium phosphate buffer.

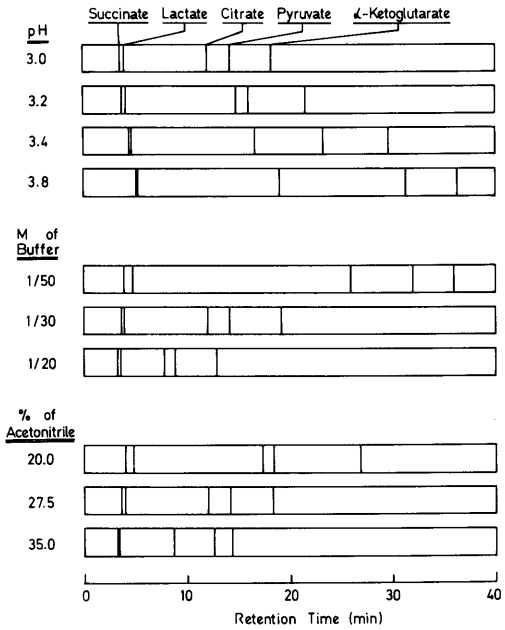

Fig. 1. Effects of pH, ionic strength, and acetonitrile concentration of the eluent on the retention time of organic acids.

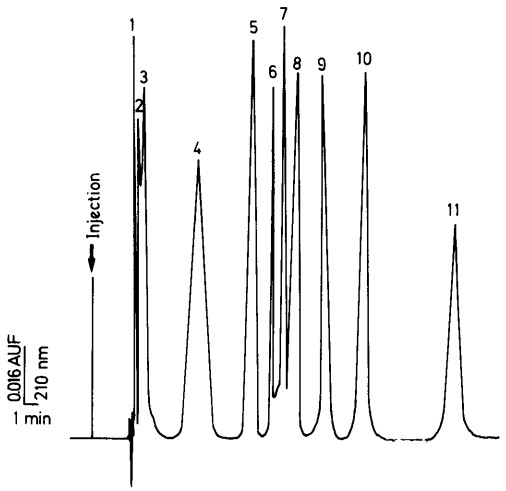

Fig. 2. Typical elution profile of organic acids on IEX 520QAE resin. Eluent, 1/30 M potassium phosphate buffer plus 27.5% acetonitrile. Column, 7.6×100mm.Sample: 1. Acetate (30 μg) 2. Succinate (400μg) 3. Lactate (50 μg) 4. Malate (80 μg) 5. Isocitrate (400 μg) 6, Citrate (80 μg) 7. Oxaloacetate (40 μg) 8. Pyruvate (5 μg) 9. α-Ketoglutarate (10 μg) 10. Fumarate (1.5 μg) 11. Succinyl CoA (100 μg).

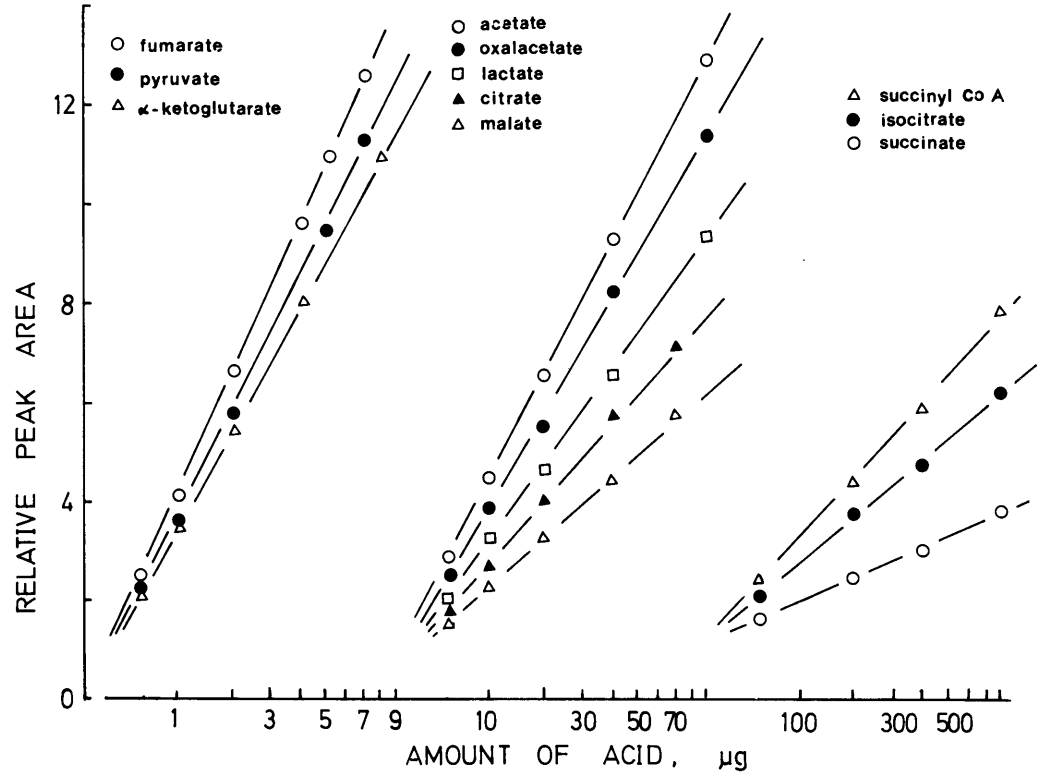

Fig. 3. Relationship between the amount of organic acids and the relative peak area.

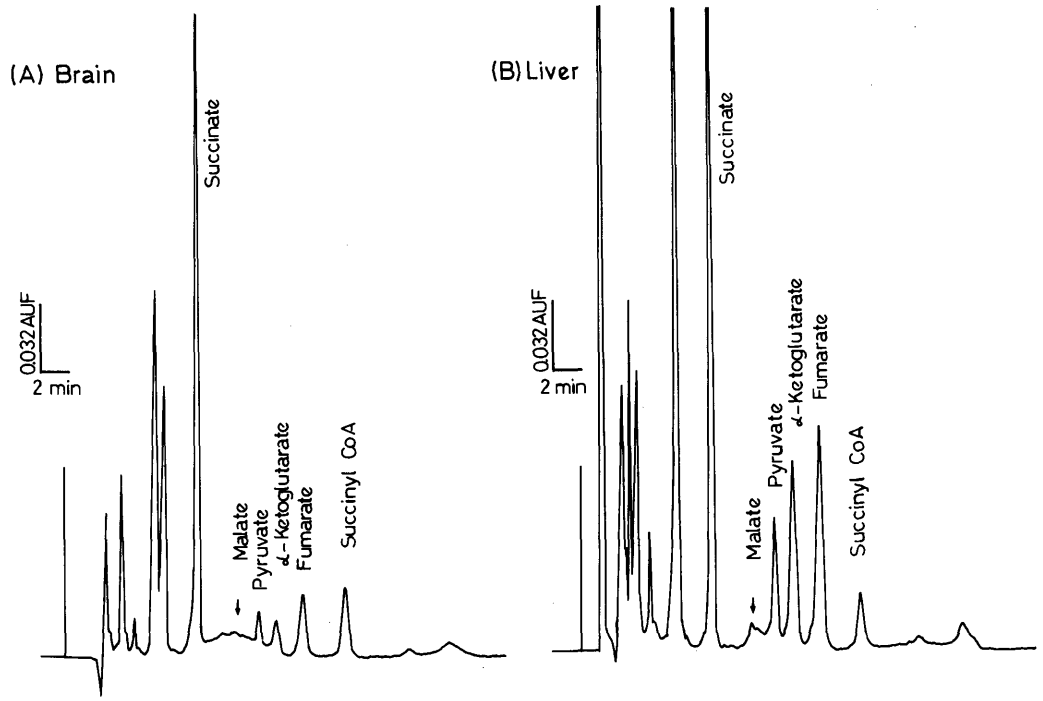

Fig. 4. Elution profile of organic acids from rat tissues.

Table 1. Amounts of acids of the citric acid cycle and related pathways in rat tissues

Acid	Brain (μmol/g tissue)	Liver (μmol/g tissue)
Succinate	189.67±16.13[a)]	129.92±17.96
Lactate	0.32± 0.08	–[b)]
Malate	0.98± 0.22	2.46± 0.11
Isocitrate	1.81± 0.43	0.74± 0.18
Citrate	0.57± 0.16	0.12± 0.05
Oxaloacetate	0.22± 0.06	0.26± 0.06
Pyruvate	0.22± 0.02	0.02± 0.01
α-Ketoglutarate	0.16± 0.02	0.33± 0.01
Fumarate	0.07± 0.01	0.07± 0.01
Succinyl CoA	1.73± 0.02	1.75± 0.01

a) Mean±S.D. of 4 experiments.
b) Not detected.

The effect of the acetonitrile concentration on the separation of some organic acids was also investigated (bottom panel, Fig. 1). When the concentration of acetionitrile was increased in the eluents, the retention times of the organic acids decreased. With 20% acetonitrile, succinate and lactate were clearly separated but their peak hights decreased. This phenomenon may be due to the partitive ability of the resin. From these results, it seems that the best eluent for the separation of acids of the citric acid cycle on a short column of IEX 520QAE is 1/30 M potassium phosphate buffer and 27.5% (v/v) acetonitrile (pH 3.0) at 40°C.

Fig. 2 shows a typical chromatogram of a prepared mixture of 11 organic acids.

The linear relationship between the peak-area response and the amount of organic acids is shown in Fig. 3. In this relationship, the sensitivity to succinate, isocitrate, and succinyl CoA was lower than that for the other organic acids, but even this lesser sensitivity was satisfactory for the analysis of condensed biological samples.

Fig. 4 shows the elution profiles of the rat brain and liver. The peak of each acid was identified by an addition of the standard. In this experiment, the retention time of each acid was slightly longer than that of the authentic standard, probably because other

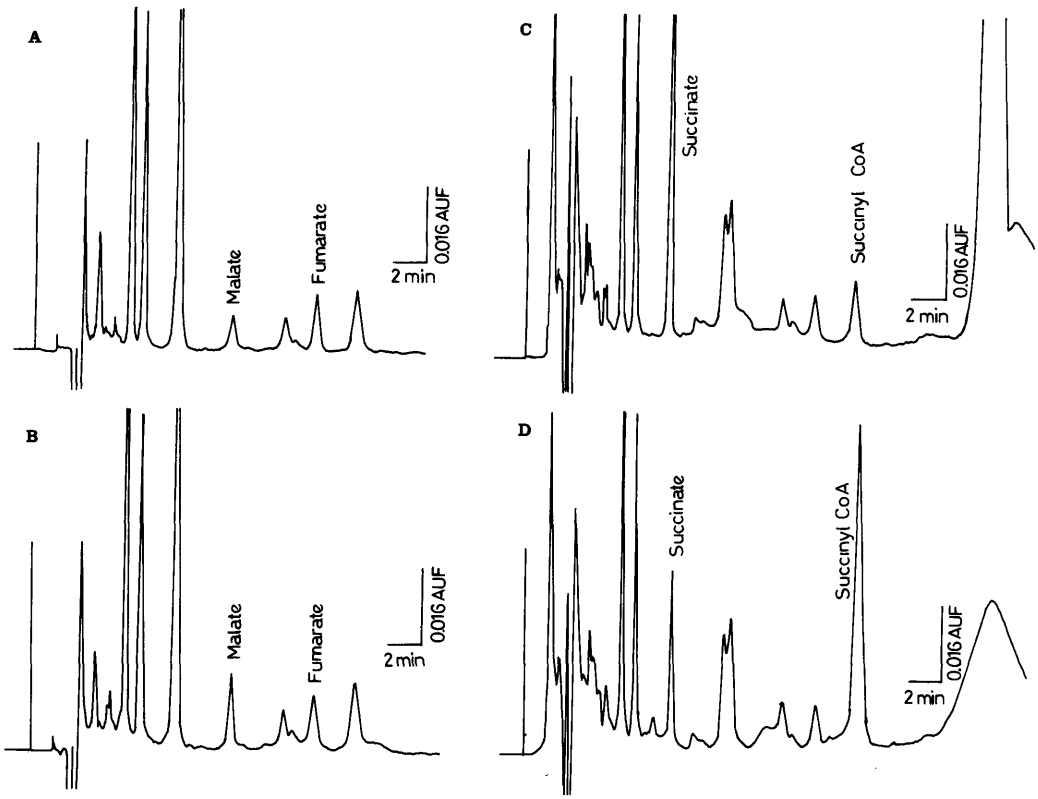

Fig. 5. Assay of enzyme reaction mixture. (A) Mixture of fumarase and rat brain extract at time zero. (B) 90 min after the start of incubation with fumarase and rat brain extract. (C) Mixture of succinic thiokinase and rat brain extract at time zero. (D) 120 min after the start of incubation with succinic thiokinase and rat brain extract.

anionic substances interfered with elution. The acids were, however, clearly separated.

Amounts of the different acids are listed in Table 1. These net values were somewhat different those given in other reports [5, 13], perhaps because of differences in the isolated method for the animal tissue or in the analytical methods.

An analysis of the acids of the citric acid cycle in the reaction mixture of an enzyme and a biological extract was also done (Fig. 5). By incubation with fumarase, the malic acid content became significantly increased and fumaric acid decreased. These changes reached an equilibrium 30 min after fumarase added. The similar changes were seen with succinic thiokinase.

DISCUSSION

Various factors are affecting the resolution. In a preliminary study, IEX 520QAE resin was packed in a 30 cm long column with a 4.6 mm diameter and used with a flow rate of 0.8 m*l*/min. As a result, the pressure was often over 100 kg/cm^2, which was critical for this hydrophilic, spherical, porous gel resin. Consequently, in this study a short column with 10 cm length and 7.6 mm diameter was used to prevent the destruction of the resin by a high pressure. At the flow rate of 1.2 m*l*/min, the pressure remained at about 5 kg/cm^2, and acetic acid emerged about 2 min after the injection.

Continuous monitoring of organic acids separated on a gel column was introduced

by Kesner and Muntwyler [10]. The effluent from their column was mixed with sodium orthonitrophenate and was measured spectrophotometrically at 350 nm. Several groups [6, 9, 17] have separated the acids of the citric acid cycle on a column containing various resins, and monitored the column effluent by UV spectrophotometry at 210 or 220 nm. UV absorption at 210 nm detects not only the acids of the citric acid cycle but also other carboxyl groups, and this monitoring system has been used to analyze a wide variety of organic acids, peptides, and nucleic acids [4, 19].

In biological samples, one of the difficulties in identifing the acids arises from contaminating substances that absorb UV at 210 nm. One way to attack the problem is to remove the contaminants from the preparation. Treatment beforehand with cationic ion-exchange chromatography was tried to remove cationic substances in the biological preparations. Since Dowex 50W-X8 absorbs most amino acids and peptides, the organic acids and other anionic substances were passed through such column, but the procedure resulted in an increase in the sample volume and was accompanied by condensation.

In conclusion, the acids of the citric acid cycle and related pathways can be separated within 30 min by high-performance ion-exchange chromatographly equipped with a short column of the anion-exchange resin IEX 520QAE.

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REFERENCES

1. Bulen, W. A., Varner, J. E., and Burrell, R. C. 1952. Separation of organic acids from plant tissues. *Anal. Chem.* 24: 187–190.
2. Busch, H., Hurlbelt, R. B., and Potter, V. R. 1952. Anion exchange chromatography of acids of the citric acid cycle. *J. Biol. Chem.* 196: 717·727.
3. Dajani, R. M., and Orten, J. M. 1957. A study of the citric acid cycle in erthrocytes. *J. Biol. Chem.* 231: 913–924.
4. Dunn, D. B., and Hall, R. H. 1957. Nucleic acid. Vol. 1. In: Handbook of biochemistry and Molecular biology (Fasman, G. D., ed.) CRC Press, Cleaveland.
5. Frohman, C. E., Orten, J. M., and Smith, A. H. 1951. Chromatographic determination of the acids of the citric acid cycle in tissues. *J. Biol. Chem.* 193: 277–283.
6. Funasaka, W., Hanai, T., and Fujimura, K. 1974. High speed liquid chromatographic separations of phthalic esters, carbohydrates, TCA organic acids and organic mercury compounds. *J. Chromatogr. Sci.* 12: 517–520.
7. Harlow, G. A., and Morman, D. H. 1964. Automatic ion exclusion-partition chromatography of acids. *Anal. Chem.* 36: 2438–2442.
8. Harmon, M. A., and Doelle, H. W. 1969. Gas chromatographic separation and determination of microquantities of the tricarboxylic acid cycle acids and related compounds. *J. Chromatogr.* 42: 157–169.
9. Hyakutake, H., Hanai, T. 1975. Further studies of practical high-speed liquid chromatographic separations of tricarboxylic acid cycle organic acids and carbohydrates. *J. Chromatogr.* 108: 385–390.
10. Kesner, L., and Muntwyler, E. 1966. Automatic determination of weak organic acids by partition column chromatograph and indicator titration. *Anal. Chem.* 38: 1164–1168.
11. Kinnory, D. S., Takeda, Y., and Greenberg, D. M. 1955. Chromatography of carboxylic acids on a silica gel column with a benzen-ether solvent system. *J. Biol. Chem.* 212: 379–383.
12. Kornberg, H. L. 1966. Tricarboxylic acid cycle. Vol. 2. In: Essay in Biochemistry (Campbell, P. N., and Greville, G. D., eds) pp1–32. Academic Press, New York.
13. Miller, M., and Shamban, A. 1977. A comparison of methods for stopping intermediary metabolish of developing rat brain. J. Neurochem. 28: 1327–1334.
14. Richards, M. 1975. Separation of Mono- and dicarboxylic acids by liquid chromatography. *J. Chromatogr.* 115: 259–261.
15. Shimomura, K., and Walton, H. F. 1965. Refractometoric column monitoring in ion exchange chromatography of carboxylic acids. *Anal. Chem.* 37: 1012–1014.
16. Stahl, K. W., Schäfer, G., and Lamprecht, W. 1972. Design of high efficiency liquid chromatograph using specific detection and its evaluation for analysis of tricarboxylic acid cycle intermediates and related compounds on a nanoequivalent scale. *J. Chromatogr. Sci.* 10: 95–102.

17. Turkelson, V. T., and Richards, M. 1978. Separation of the citric acid cycle acids by liquid chromatography. *Anal. Chem.* 50: 1420–1423.
18. Walter, M., and Ramaley, L. 1973. Purification of Acetonitrile. *Anal. Chem.* 45: 165–166.
19. Webster, G. C. 1970. Comparison of direct spectrophotometric methods for the measurement of protein concentration. *Biochim. Biophys. Acta* 207: 371–373.
20. Williamson, J. R., and Corkey, B. E. 1969. Assays of intermediates of the citric acid cycle and related compounds by fluorometric enzyme methods. ææ434–513. In: Methods in Enzymology. (Lowenstein, J. M., ed) Vol. 13. Academic Press, New York.

要　約

動物組織のクエン酸回路の酸の高速液体クロマトグラフィーによる分離：津山伸吾（大阪府立大学農学部家畜生理学教室）——クエン酸回路及び関連代謝系の有機酸の分離，定量について，陰イオン交換樹脂を用いた高速液体クロマトグラフィー法を開発し，ラット脳・肝臓に含まれるクエン酸回路の有機酸及び同回路の酸素反応液の生成物や基質の定量に用いた。強陰イオン交換樹脂 IEX 520 QAE を充填した短カラムを40℃に維持し27.5%アセトニトリルを含む1/30M 燐酸緩衝液（pH3.0）により溶離，210nm における吸収を連続的にモニターした。