Journal of Chromatography, 227 (1982) 1—9
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1065

CHANGES OF ORGANIC ACIDS IN RAT HEART MUSCLE UNDER ISCHEMIC-LIKE CONDITIONS

SHIN-ICHI HARAGUCHI* and HIRONORI TOSHIMA

The 3rd Department of Internal Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume-city, Fukuoka-ken, 830 (Japan)

and

ISAMU MATSUMOTO, TOMIKO KUHARA and TOSHIHIRO SHINKA

Research Institute of Medical Mass Spectrometry, Kurume University School of Medicine, 67 Asahi-machi, Kurume-city, Fukuoka-ken, 830 (Japan)

(First received May 27th, 1981; revised manuscript received August 12th, 1981)

SUMMARY

Gas chromatographic—mass spectrometric analysis demonstrated the presence of organic acids such as lactic acid, glycolic acid, compounds related to the tricarboxylic acid cycle, fatty acids, and deoxyaldonic acids in rat heart muscle. The variation of these organic acids was examined over a range of time elapsed after decapitation. The results showed that lactic acid, glycolic acid and deoxyaldonic acids of 3-deoxy-2-C-(hydroxymethyl)-tetronic acid, 3-deoxyerythropentonic acid and 3-deoxy-2-C-(hydroxymethyl)erythro-pentonic acid increased until 4 min after decapitation, but then decreased from 6 min after decapitation. On the other hand, 2-deoxytetronic acid and dideoxypentonic acid markedly increased and unknown peaks appeared on the gas chromatogram from 6 min after decapitation.

INTRODUCTION

Research into heart metabolism has primarily been concerned with energy metabolism. Organic acids, such as lactic acid, pyruvic acid, the tricarboxylic acid cycle-related compounds α-ketoglutaric acid or citric acid, and fatty acids, have been determined in the coronary blood or in the heart muscle of the rat or in patients with ischemic heart disease [1—4]. Research on organic acids that are not related to energy metabolism has been relatively uncommon, which is due, in part, to the difficulty in developing satisfactory quantitative techniques.

0378-4347/82/0000—0000/$02.75

With recent developments in the application of gas chromatography—mass spectrometry (GC—MS) to the medical sciences, it is now possible to analyze simultaneously many compounds in a metabolic system, thus providing an efficient method for investigating the whole metabolic system in question.

We have therefore examined the constituents of organic acids and their related metabolites in normal rat heart muscle after decapitation, using GC—MS. The variation of the organic acids, particularly deoxyaldonic acids, in rat heart muscle under pathological conditions was investigated.

MATERIALS AND METHODS

Chemicals

Lactic acid, glycolic acid, glycerol, succinic acid, fumaric acid, palmitic acid and stearic acid were commercial products. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Tokyo Kasei Co. (Tokyo, Japan). All other reagents were of the highest purity available commercially.

Gas chromatography and gas chromatography—mass spectrometry

A Shimadzu GC-6A gas chromatograph with dual flame ionization detectors was used. A glass coiled column (2 m × 3 mm I.D.) was packed with 3% OV-17 on Gas-Chrom Q (80—100 mesh). The column oven was maintained isothermally at 80°C for 2 min and then programmed at 6°C/min until 290°C. Peak areas and retention times were determined with an on-line Shimadzu Chromatopac 4-B computer equipped with a printer. For identification of the compounds, a JEOL JMS-D 100 GC—MS system with an on-line JMA 2000 data acquisition system was used.

Mass spectra were recorded at an ionizing voltage of 75 eV with a 300-μA trap current, and ion source temperature of 280°C. The magnet of the mass spectrometer was scanned repetitively over field strengths from *m/z* 50 to *m/z* 700 every 5 sec.

Sample preparation

Rats (250—300 g) of the Sprague—Dawley strain bred with commercial foods and water ad libitum were decapitated, and their hearts were immediately excised and frozen in dry ice—acetone. In order to examine the variation of the organic acids with time elapsed after decapitation, hearts were obtained at 2, 4, 6, 10, 15 and 30 min, and then frozen. One hundred milligrams were removed from each specimen, minced with scissors in cold saline solution and homogenized with 500 μl of the saline solution using a Potter homogenizer. Prior to deproteinization with 3 ml of 99% cold ethanol, protein in the heart muscle was determined by the Bio-Rad protein assay method. Heptadecanoic acid (20 μg/mg of protein) was added to the homogenate as an internal standard. The samples were centrifuged at 25,000 *g* for 10 min. The precipitate was washed once with cold ethanol to extract any remaining soluble material and centrifuged again. The second supernatant was combined with the first supernatant.

The supernatant of each ethanol-treated specimen was concentrated to 0.5 ml to remove the ethanol. Distilled water, 3 ml, was added to the residue.

The solution was acidified to pH 1 with 2 *N* HCl. Organic acid fractions were obtained by extraction with equal volumes of diethyl ether and ethyl acetate twice. Organic solvent extracts were dried over anhydrous sodium sulphate for 1—2 h. The extracts were concentrated to 1 ml using an evaporator and then dried under a stream of nitrogen.

The samples were trimethylsilylated by adding 200 μl of BSTFA to the residue; the mixtures were then heated to 60°C for 1 h in glass tubes with PTFE-covered screw caps. Aliquots of the samples were subjected to GC and GC—MS analysis.

RESULTS

The profile of organic acids in 100 mg of rat heart muscle is shown in Fig. 1. Over 40 peaks were detected on the gas chromatogram. The peak

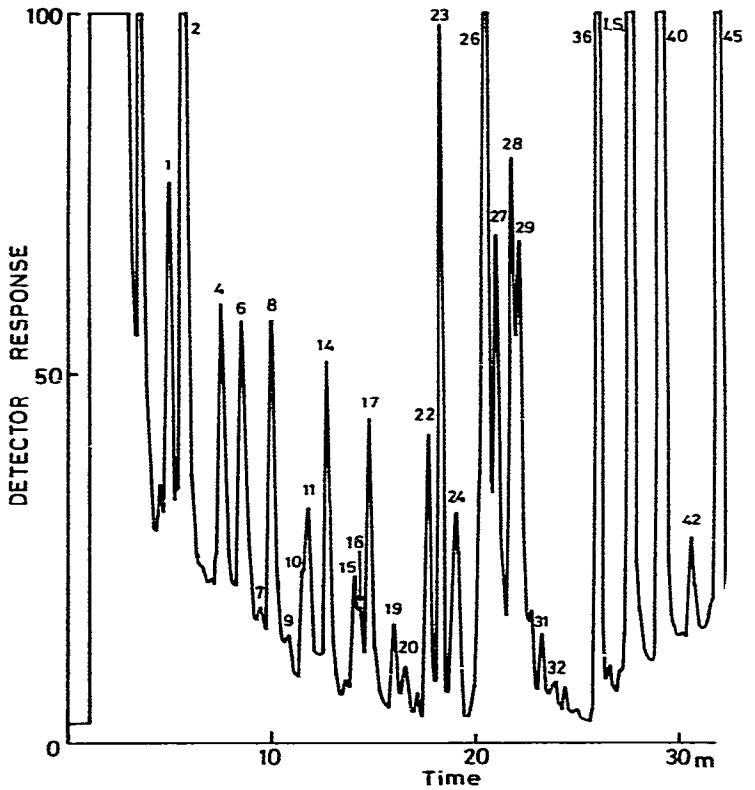

Fig. 1. Gas chromatogram of trimethylsilyl (TMS) derivatives of organic acids obtained from 100 mg of rat heart muscle, excised and frozen within 30 sec. The peaks were identified as follows: (1) lactic acid, (2) glycolic acid, (4) 3-hydroxypropionic acid, (7) glycerol, (9) 2-methylglyceric acid, (10) phosphoric acid, (11) glyceric acid, (14) succinic acid + fumaric acid, (15) 3-deoxytetronic acid, (16) 2-deoxytetronic acid, (17) 3-deoxy-2-C-(hydroxymethyl)tetrono-1,4-lactone, (19) malic acid, (20) dideoxypentonic acid, (22) 3-deoxy-2-C-(hydroxymethyl)tetronic acid, (23) 3-deoxypentono-1,4-lactone, (24) 3-deoxy-erythropentonic acid, (26) 3-deoxy-2-C-(hydroxymethyl)pentono-1,4-lactone, (27) β-glycerophosphoric acid, (28) α-glycerophosphoric acid + 3-deoxy-2-C-(hydrocymethyl)-erythropentonic acid, (36) palmitic acid, (40) stearic acid.

which appears between peaks 36 and 40 is the internal standard, heptadecanoic acid. The ratio of each peak area to that of the internal standard was calculated, and the deviations of each peak from ten rat heart muscles were examined. The deviations of all peaks except peaks 6 and 27 were within 100% (data not shown). The profile of organic acids in rat heart muscle is therefore relatively stable.

Identification of these peaks was performed by comparing their mass spectra and retention times with those of laboratory samples or from literature references. A mass spectrum obtained from peak 24 is shown as an example (Fig. 2). The molecular ion at *m/z* 438 was not detected but ion at *m/z* 423 (M — 15) was found as a relatively small peak. Other fragment ions, ion at *m/z* 333 (M — 15 — 90), ion at *m/z* 245 (335 — 90) and at *m/z* 231 (321 — 90), were detected. The base peak was observed at *m/z* 73. This mass spectrum and the retention time were consistent with that previously reported [5]. Peak 24 was, therefore, identified as 3-deoxyerythropentonic acid tetra-trimethylsilyl derivative. In this way, each peak was identified and detection of various deoxyaldonic acids were made. The compounds identified are shown in the legend of Fig. 1.

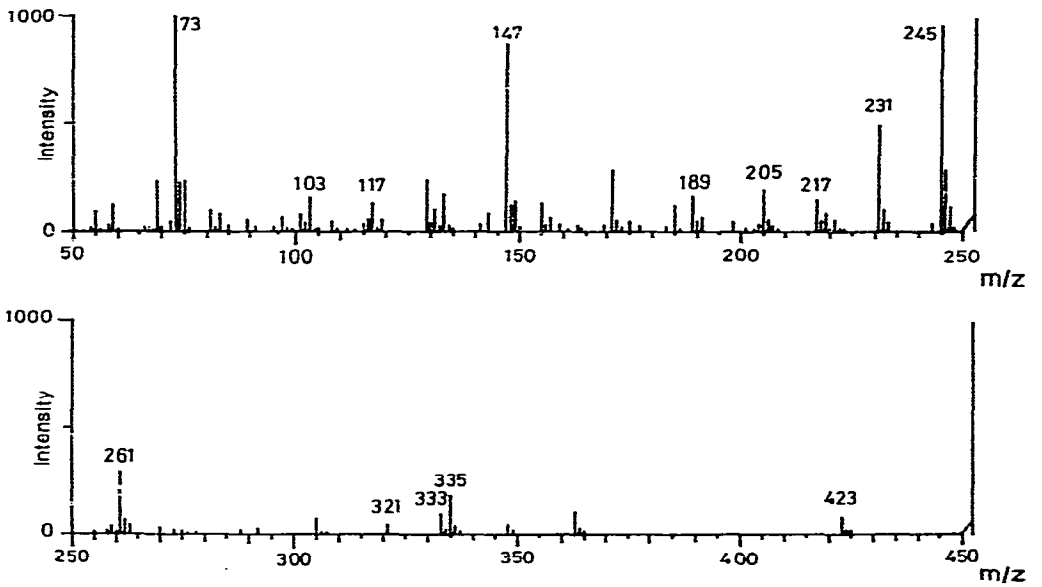

Fig. 2. Mass spectrum of the TMS derivative of 3-deoxyerythropentonic acid, which was obtained from peak 24 in Fig. 1.

The variation of these organic acids detected in rat heart muscle was examined by the time elapsed after decapitation to determine the metabolic change. The gas chromatogram of organic acids detected in heart muscle obtained 4 min after decapitation is shown in Fig. 3. Almost all peaks were increased in intensity compared with the control (analyzed within 30 sec of decapitation), especially glycolic acid, 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid and fatty acids (as indicated by arrows).

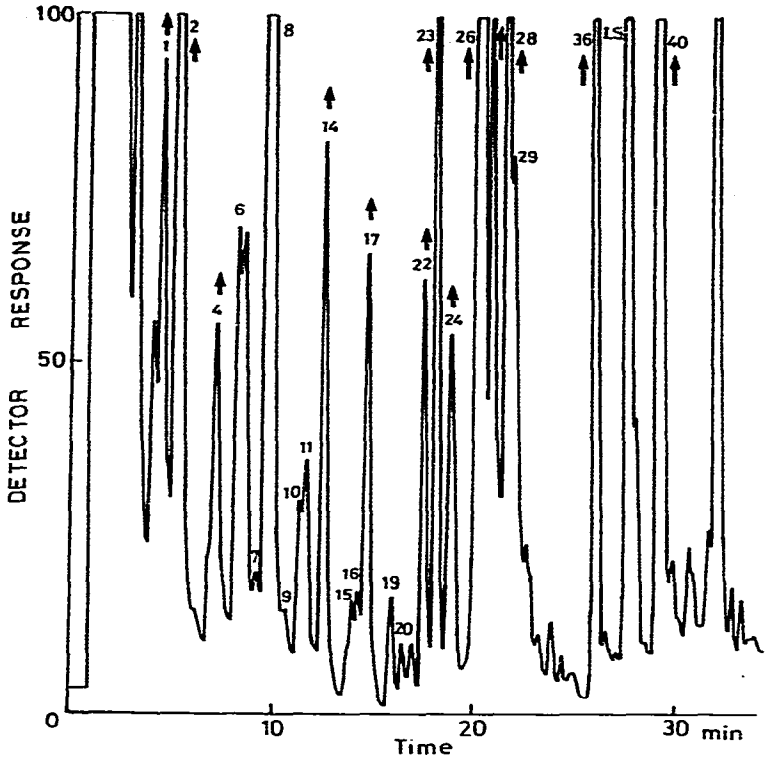

Fig. 3. Gas chromatogram of TMS derivatives of organic acids from 100 mg of rat heart muscle excised and frozen at 4 min after decapitation. The peak numbers in the figure correspond to those in Fig. 1.

It was observed that the heart had virtually stopped beating and coronary flow was at a standstill 6 min after decapitation.

The gas chromatogram obtained from the experiment of 10 min elapsed is shown in Fig. 4. Most peaks including 3-deoxy-2-C-(hydroxymethyl)tetronic acid, 3-deoxyerythropentonic acid and 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid (as indicated by arrows) had decreased when compared to Fig. 1. Only two deoxyaldonic acids, 2-deoxytetronic acid (peak 16) and dideoxypentonic acid (peak 20), however, had markedly increased; in addition, unknown peaks appeared at the position of peaks 24, 28, 32 and 36. The mass spectra of these unknown peaks have almost the same fragment ions but different molecular ions with differences of 74 mass units, i.e. M^+ = 444, 518, 592 and 666. These data suggest that the unknown peaks may be homologous compounds.

The variation of these deoxyaldonic acids with time elapsed after decapitation is tabulated in Fig. 5. It can be seen that the three deoxyaldonic acids — 3-deoxy-2-C-(hydroxymethyl)tetronic acid, 3-deoxyerythropentonic acid and 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid — increased in heart muscle until 4 min, but decreased markedly from 6 min after decapitation. Both 2-deoxytetronic acid and dideoxypentonic acid, however, increased from 6 min elapsed after decapitation.

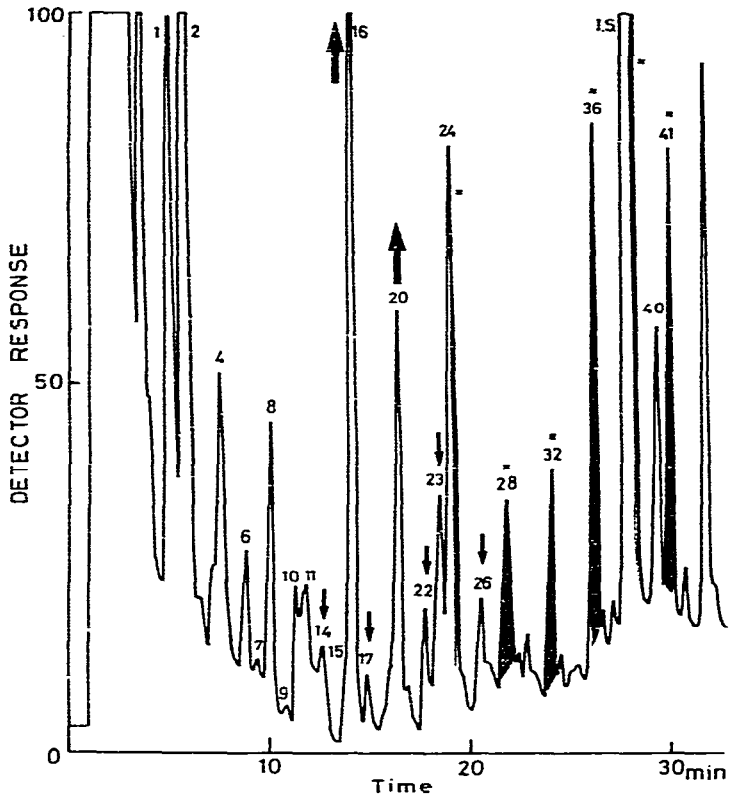

Fig. 4. Gas chromatogram of TMS derivatives of organic acids from 100 mg of rat heart muscle excised and frozen at 10 min after decapitation. The peak numbers in this figure correspond to those in Figs. 1 and 3.

DISCUSSION

GC—MS has been used successfully in profiling organic acids in various fluids and tissues (brain and heart muscle), and to identify compounds which are well separated by a packed column. More peaks would be separated by using a capillary column, but it is not suitable for profiling because of poor reproducibility. Previously profiling of organic acids has been successfully performed using a packed column [6—8]. The packed column was therefore used here for the separation of organic acids in heart muscle.

It is well known that heart muscle utilizes fatty acids in preference to glucose as an energy source. The evidence that palmitic acid and stearic acid were detected in large amounts on the gas chromatogram, further confirms the importance of fatty acids in the energy metabolism of heart muscle.

Deoxyaldonic acids have been detected by GC—MS analysis in biological fluids such as serum, urine and amniotic fluid, as well as in rat brain tissue [9—11]. The detection of the deoxyaldonic acids 3-deoxytetronic acid, 2-deoxytetronic acid, dideoxypentonic acid, 3-deoxy-2-C-(hydroxymethyl)-tetronic acid, 3-deoxyerythropentonic acid, 3-deoxy-2-C-(hydroxymethyl)-erythropentonic acid and their lactones in heart muscle has been reported

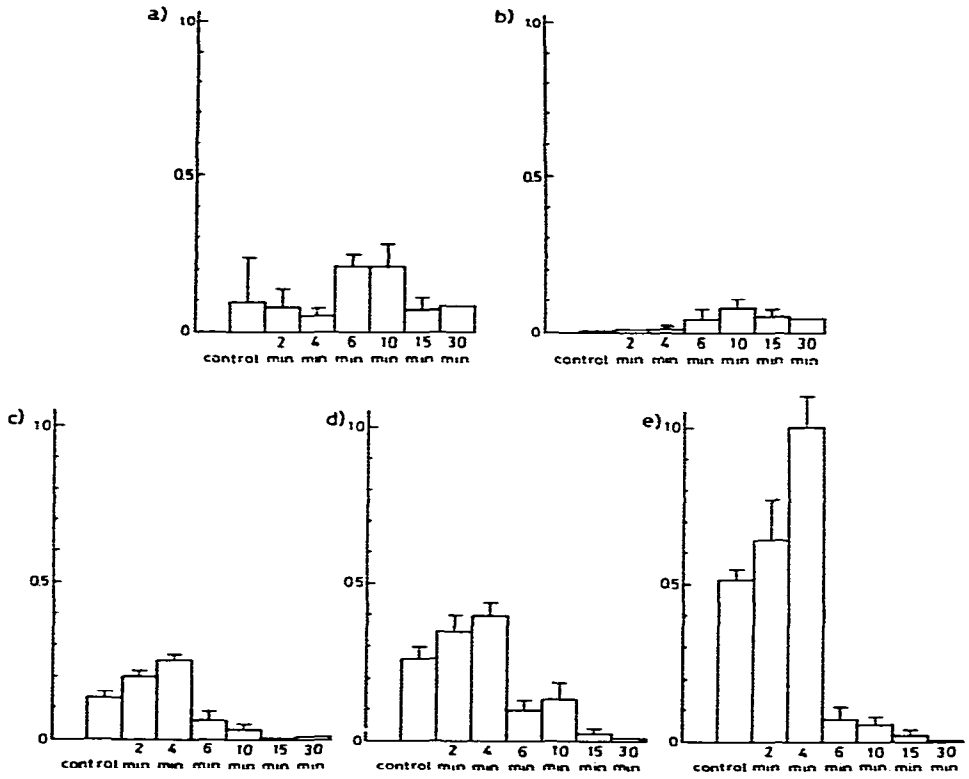

Fig. 5. The changes in deoxyaldonic acids after decapitation. Each bar represents data averaged from three specimens. The abscissa represents 2 min, 4 min, 6 min, 10 min, 15 min and 30 min time elapsed, and the ordinate represents peak area ratio with respect to an internal standard. (a) 2-Deoxytetronic acid, (b) dideoxypentonic acid, (c) 3-deoxy-2-C-(hydroxymethyl)tetronic acid, (d) 3-deoxyerythropentonic acid, (e) 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid.

[12]. Neither the function nor the metabolism of deoxyaldonic acids, however, is clearly understood. Fell et al. [13] have recently determined 2-deoxytetronic acid in urine and plasma. They recommended that alkaline conditions should be avoided during sample preparation because 2-deoxytetronic acid can be formed by the action of alkali on certain sugars such as maltose. As our extraction procedure was not performed under basic conditions, we believe that 2-deoxytetronic acid in heart muscle is a bona fide metabolite and not an artifact of sample preparation.

Lawson et al. [6] noted that urinary excretion of 2-deoxytetronic acid changed more than that of the other deoxytetronic acids in an experiment in which a high glucose diet was given. They therefore concluded that the immediate metabolic precursors of this compound are more closely related to glucose than are those of the other deoxytetronic acids. Fell et al. also indicated that 2-deoxytetronic acid in urine may largely result from chemical degradation of body carbohydrate and may possibly be a metabolite of γ-hydroxybutyric acid by β-oxidation. Lawson et al. [6] considered that as the amounts of aldonic acid and deoxyaldonic acid found in urine, other

than 2-deoxytetronic acid, change little in association with large fluctuations in the composition of the diet, they may be mainly endogenous. It is known that oxidation of pentoses and deoxypentoses may yield pentonic and 2-deoxypentonic acids, and ascorbic acid is metabolized via threonic acid as well as via L-lyxonic acid and L-xylonic acid (pentonic acid). It is therefore presumed that certain deoxyaldonic acids detected in rat heart muscle may be derived from carbohydrate or be concerned with glucose metabolism, especially from the pentose phosphate shunt, although this has not been elucidated. However, little is known about the origin of 3-deoxy-2-C-(hydroxymethyl)aldonic groups at present.

Lactonization is a potential problem when measuring acids that contain a hydroxy group at the 4 carbon position. As Horning and Horning [7] identified the tetronic acid lactones in solvent (ethyl acetate and ether) extracts of acidified urine and Thompson et al. [8] noted that lactones were formed under acidic conditions, it is believed that lactones detected in heart muscle are also formed under acidic conditions, with which the extraction of organic acids was performed.

It is presumed that the supply of oxygen and coronary blood flow are severely reduced at 4 min after decapitation, although specific measurements were not made. Lactic acid and glycolic acid were increased, and it was observed that the heart was remarkably bradycardic at the time. It is reasonable to assume, therefore, that the whole heart muscle was in an ischemic condition at this time lapse, although this experimental ischemic condition is different from regional ischemia, so-called "ischemic heart disease". The peak of glycolic acid is always detected as a larger peak than that of lactic acid, which is regarded as one of the indexes of ischemia. This suggests that glycolic acid may also be an index of ischemic heart, as it changes in parallel to lactic acid. It is presumed that glycolic acid may be formed from glyoxylic acid by the same kind of enzymatic reaction as that of lactate formation. It was observed that peaks of deoxyaldonic acids of 3-deoxy-2-C-(hydroxymethyl)tetronic acid, 3-deoxyerythropentonic acid and 3-deoxy-2-C-(hydroxymethyl)erythropentonic acid were increased at 4 min time lapse. These results suggest that these three deoxyaldonic acids may also accumulate in ischemic heart muscle, although the degree of hypoxia and ischemia was not precisely determined.

It was observed that the heart stopped beating and coronary flow was at a standstill 6 min after decapitation. Two aldonic acids — 2-deoxytetronic acid and dideoxypentonic acid — had clearly increased, the three deoxyaldonic acids mentioned above decreased significantly and unknown peaks appeared. Although the results can not be fully explained at present, it may be of value in the future to understand why 2-deoxytetronic acid and dideoxypentonic acid temporarily accumulate, and to determine the unidentified compounds produced in early dead (infarcted) heart muscle.

It is presumed, however, that the cause of the variation in these deoxyaldonic acids resulting from reduced coronary blood flow might be related to glucose metabolism. As the metabolism of deoxyaldonic acid has not yet been elucidated, we await future research in this field.

ACKNOWLEDGEMENTS

This study was supported by a Research Grant for the Intractable Diseases from the Ministry of Health and Welfare of Japan. English correction of this manuscript by Dr. Yong Y. Lin is appreciated.

REFERENCES

1 R.J. Bing, Physiol. Rev., 45 (1965) 171.
2 J.F. Oram, J.I. Wenger and J.R. Neely, J. Biol. Chem., 250 (1975) 73.
3 L.H. Opie, Jap. Circ. J., 42 (1978) 1223.
4 L.H. Opie, Amer. Heart J., 97 (1979) 375.
5 C. Petersson, Tetrahedron, 26 (1970) 3413.
6 A.M. Lawson, R.A. Chalmers and W.E. Watts, Clin. Chem., 22 (1976) 1283.
7 E.C. Horning and M.G. Horning, Clin. Chem., 17 (1971) 802.
8 J.A. Thompson, S.P. Markey and P.V. Fennessey, Clin. Chem., 21 (1975) 1892.
9 K. Mitsutake, T. Shinka, T. Kuhara, I. Matsumoto, K. Kabashima, H. Abe and T. Kato, in B. Munson (Editor), 28th Annual Conference on Mass Spectrometry and Allied Topics, New York, May 25—30, 1980, p. 288.
10 T. Sakata, Y. Oomura, M. Fukushima, K. Tsutsui, K. Hashimoto, T. Kuhara and I. Matsumoto, Brain Res. Bull., 5 (1980) 23.
11 S. Yamada, T. Kuhara, I. Matsumoto and K. Inanaga, Jap. Soc. Med. Mass Spectrom., 4 (1979) 293.
12 S. Haraguchi, H. Toshima, T. Kuhara, T. Shinka and I. Matsumoto, in B. Munson (Editor), 28th Annual Conference on Mass Spectrometry and Allied Topics, New York, May 25—30, 1980, p. 279.
13 V. Fell, C.R. Lee and R.J. Pollitt, Biochem. Med., 13 (1975) 40.