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# ISOTACHOPHORESIS IN TWO-DIMENSIONAL COMBINATION WITH ZONE ELECTROPHORESIS FOR THE CONCENTRATION AND SEPARATION OF GLUCOSE METABOLITES

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SUMMARY

Isotachophoresis and zone electrophoresis were used separately or in combination for the separation of a number of substances. Metabolites synthesized from radioactive precursors were extracted isotachophoretically from whole blood or muscle tissue.

## INTRODUCTION

In a preceding paper[1], isotachophoresis, either alone or in two-dimensional combination with zone electrophoresis, was used for the isolation of some labelled phosphate esters. For many mixtures of ions, the one-dimensional technique gave an adequate degree of resolution. For more complex mixtures, the two-dimensional combination of isotachophoresis with zone electrophoresis was used. No pretreatment of the samples was necessary (de-proteination or concentration). During the isotachophoretic procedure, ionic species that occur even at very low concentrations in the samples were concentrated to sharp zones of high concentration. In this paper, the use of the same techniques for the concentration and separation of a number of glucose metabolites is described.

## MATERIALS

Unlabelled phosphate esters and enzyme preparations were obtained from Sigma Chemical Co. or Boehringer & Söhne GmbH, G.F.R. Most of the radioactive substances were obtained from the Radiochemical Centre, Amersham, Great Britain, and some others were synthesized. [1-$^{32}$P]1,3-diphosphoglycerate ([1-$^{32}$P]-1,3-DPG) was prepared by oxidizing glyceraldehyde-3-phosphate (GAP) in the presence of inorganic [$^{32}$P]phosphate ($^{32}P_i$), NADP and glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.13)[2]. [1,2,3-$^{14}$C]Sedoheptulose-1,7-diphosphate (SDP) and [1,2,3-$^{14}$C]octulos-1,8-diphosphate (ODP) were condensation products of [U-$^{14}$C] dihydroxy acetone phosphate (DHAP) with erythrose-4-phosphate (E4P) or ribose-5-phosphate (R5P), respectively, formed in the presence of muscle aldolase (E.C. 4.1.2b)[3,4]. [U-$^{14}$C]Ribulose-5-phosphate (Ru5P), [U-$^{14}$C]-6-phosphogluconate

(6PG) and [U-$^{14}$C]ribulose-1,5-diphosphate (RuDP) were prepared as described previously[1]. [U-$^{14}$C]R5P was obtained when Ru5P was incubated with phosphoriboisomerase (E.C. 5.3.1.6)[5].

The enzymatically synthesized substances were isolated either by one-dimensional isotachophoresis (systems I and II, see Table I) or by isotachophoresis in two-dimensional combination with zone electrophoresis (system V), as described earlier[1].

## METHODS

The isotachophoretic and zone electrophoretic techniques described earlier were used[1]. For the two-dimensional separations, isotachophoresis (first direction) was run on 0.4 × 20 cm cellulose acetate strips and zone electrophoresis (second direction) on 16.5 × 21 cm cellulose thin-layer plates. The electrolytes used for sotachophoresis and zone electrophoresis are summarized in Table I.

TABLE I

ELECTROLYTES USED FOR ISOTACHOPHORESIS AND ZONE ELECTROPHORESIS

| *Design* | *Electrolyte system* | *Concentration (M)* | *pH* | *Cathode vessel* | *Anode vessel* | *Strip or plate* | *Direction* |
|---|---|---|---|---|---|---|---|
| I | Tris–HCl | 0.2 | 7.2 | | + | + | 1 |
| II | Tris–HCl | 0.4 | 7.2 | | + | + | 1 |
| III | Tris–ascorbate | 0.2 | 7.2 | + | | | 1 |
| IV | Ammonium formate | 0.2 | 3.75 | + | + | + | 2 |
| V | Ammonium formate | 0.4 | 3.75 | + | + | + | 2 |
| VI | Sodium EDTA | 0.2 | 7.4 | + | + | + | 2 |

Isotachophoresis and zone electrophoresis were carried out with various glucose metabolites. In the isotachophoretic experiments, the substances were grouped according to their mobilities in relation to the mobilities of four coloured reference substances. One of the reference substances, the blue indigotetrasulphonate (t), was added to the samples before the experiments. The three other reference substances, $Y_1$, $Y_2$ and $Y_3$, all yellow, were impurities in the cellulose acetate strips. They concentrated to sharp bands during the experiments (Fig. 1). In zone electrophoresis, $P_1$ was the reference substance.


Fig. 1. Substances classed into five groups according to their mobilities in relation to the mobilities of the coloured reference substances $Y_1$, $Y_2$, $Y_3$ and indigotetrasulphonate (Table II). The area between $Y_1$ and the cathode was termed zone a; between $Y_2$ and $Y_1$, zone b; between $Y_2$ and $Y_3$, zone c; between indigotetrasulphonate (t) and $Y_3$, zone d; and between t and the anode, zone e.

For the localization of unlabelled phosphate esters, a sulphosalicylate–ferrichloride spray reagent was used[6]. Labelled compounds were localized autoradiographically.

In order to achieve mixtures that contained a number of glucose metabolites, two incubations were performed. In one of the experiments, [U-$^{14}$C]lactate was incubated together with a small piece of a muscle, which, after the incubation, acted as the sample and was placed on the wedge-shaped piece of filter-paper in the electrophoresis apparatus. In another experiment, [U-$^{14}$C]-labelled glucose was incubated with heparinized human blood. The whole incubation mixture was used as the sample.

TABLE II

RESULTS OF ZONE ELECTROPHORESIS AND ISOTACHOPHORESIS EXPERIMENTS

| *Compound* | *Zone electrophoresis* | | *Isotachophoresis* |
|---|---|---|---|
| | *System VI* $R_{Pi}$ | *System V* $R_{Pi}$ | *System I + III* *Zone* |
| Fumarate | 1.29–1.31 | 1.31 | |
| Malate | 1.23–1.24 | 0.88 | |
| Succinate | 1.24 | 0.50 | |
| α-Ketoglutarate | 1.21 | 0.94 (I)<br>1.04 (II) | |
| Citrate | 1.14–1.17 | 0.85 | |
| PEP | 1.15–1.16 | 1.22 | |
| Pyruvate | 1.07–1.09 | 1.19 | |
| 3-PGA | 1.03–1.06 | 1.18 | c |
| 2-PGA | 0.99–1.03 | 1.14 | |
| 2,3-DPG | 0.92–0.96 | 1.40–1.43 | b |
| RuDP | 0.86–0.88 | 0.92 | b |
| 1,3-DPG | 0.85–0.87 | | $Y_2$ |
| Lactate | 0.83–0.86 | 0.73 | |
| FDP | 0.83–0.84 | 0.87–0.90 | b |
| 6PG | 0.77–0.79 | 0.84 | $Y_2$ |
| SDP | 0.77–0.78 | | b |
| G3P | 0.73–0.76 | 0.82 | |
| DHAP | 0.73–0.76 | 0.80 | $Y_2$ |
| ODP | 0.72–0.75 | 0.77 | $Y_1$ |
| β-OH-butyrate | 0.71–0.74 | 0.29 | |
| E-4-P | 0.70 | 0.77 | |
| GAP | 0.63 | 0.74 | a |
| R-5-P | 0.57–0.58 | 0.69 | a |
| Ru-5-P | 0.57–0.58 | 0.64 | a |
| F-1-P | 0.49 | 0.63 | a |
| F-6-P | 0.49 | 0.61 | a |
| G-6-P | 0.49 | 0.61 | a |
| S-7-P | 0.47 | 0.56 | a |
| *Nucleotides* | | | |
| ATP | 0.47–0.49 | 0.70 | $Y_1$ |
| ADP | 0.42 | 0.50 | a |
| AMP | 0.26 | 0.30 | a |
| *Other substances* | | | |
| $P_i$ | 1.00 | 1.00 | $Y_2$ |
| Glycerate | 0.62 | 0.85 | c |

## RESULTS

In Table II, the results of the zone electrophoretic and isotachophoretic runs are presented separately. The positions of the spots in two-dimensional separations of mixtures of substances together with positions constructed from one-dimensional runs are shown in Fig. 2. The separations of the labelled compounds produced by

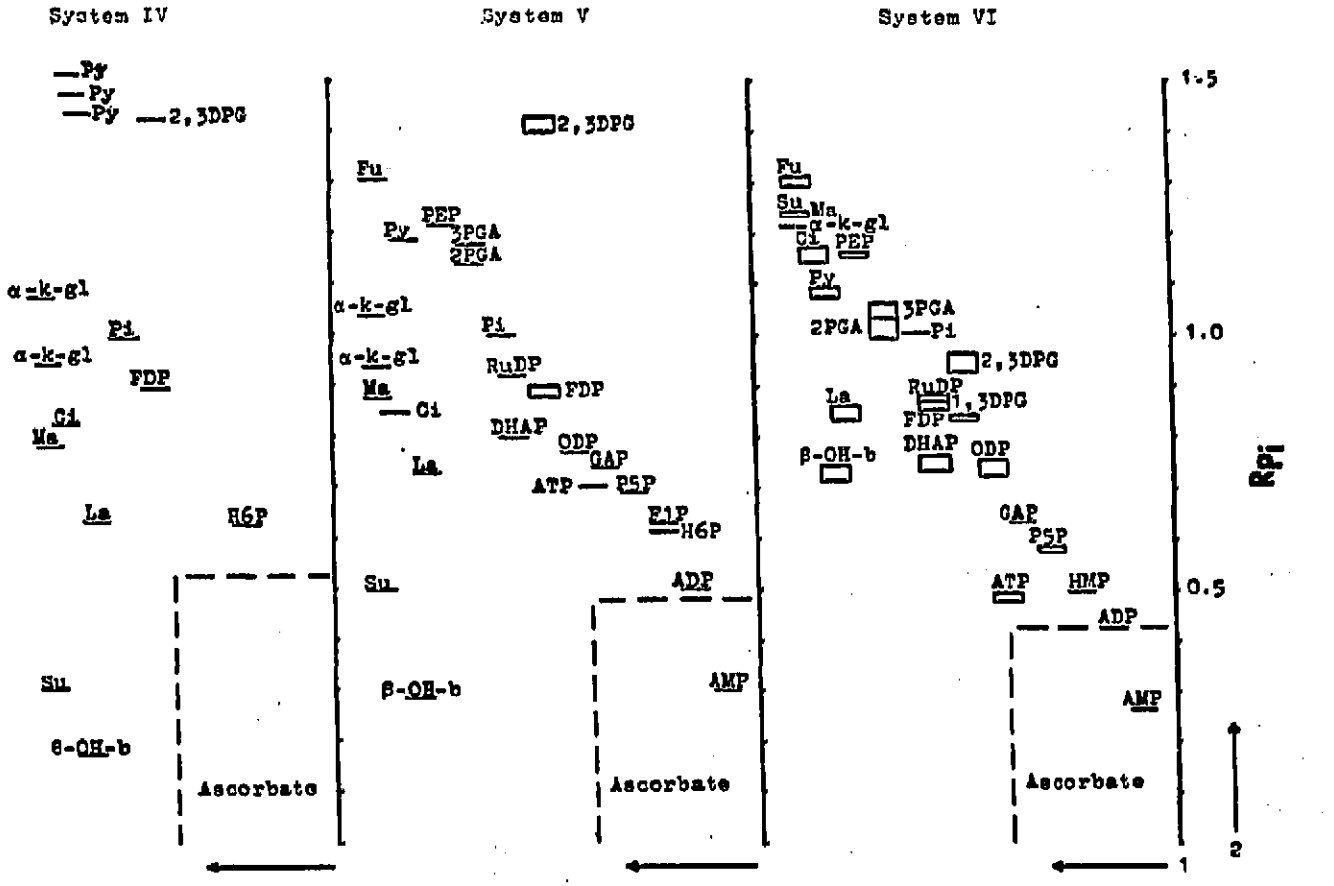


Fig. 2. Two-dimensional combinations of isotachophoresis (first dimension) and zone electrophoresis (second dimension). Electrolyte system used in the first dimension: 0.4 *M* Tris–chloride, pH 7.2, and Tris–ascorbate 0.2 *M*, pH 7.2. Zone electrophoretic systems: IV, ammonium formate, 0.2 *M*, pH 3.75; V, ammonium formate, 0.4 *M*, pH 3.75; VI, sodium EDTA, 0.2 *M*, pH 7.4.

running the incubations in the presence of muscle tissue or blood are shown in Fig. 3. The influence of the amount of citrate present and of the concentration of the leading electrolyte on the distance between lactate and pyruvate is shown in Figs. 4 and 5.

## DISCUSSION

According to the principle of isotachophoresis, ions are arranged into consecutive zones, each zone containing only one ionic species and the corresponding counter ion species[1,7,8]. An ion of high mobility, which for some reason has been left behind in a zone of low mobility, will soon overtake its slower moving neighbours

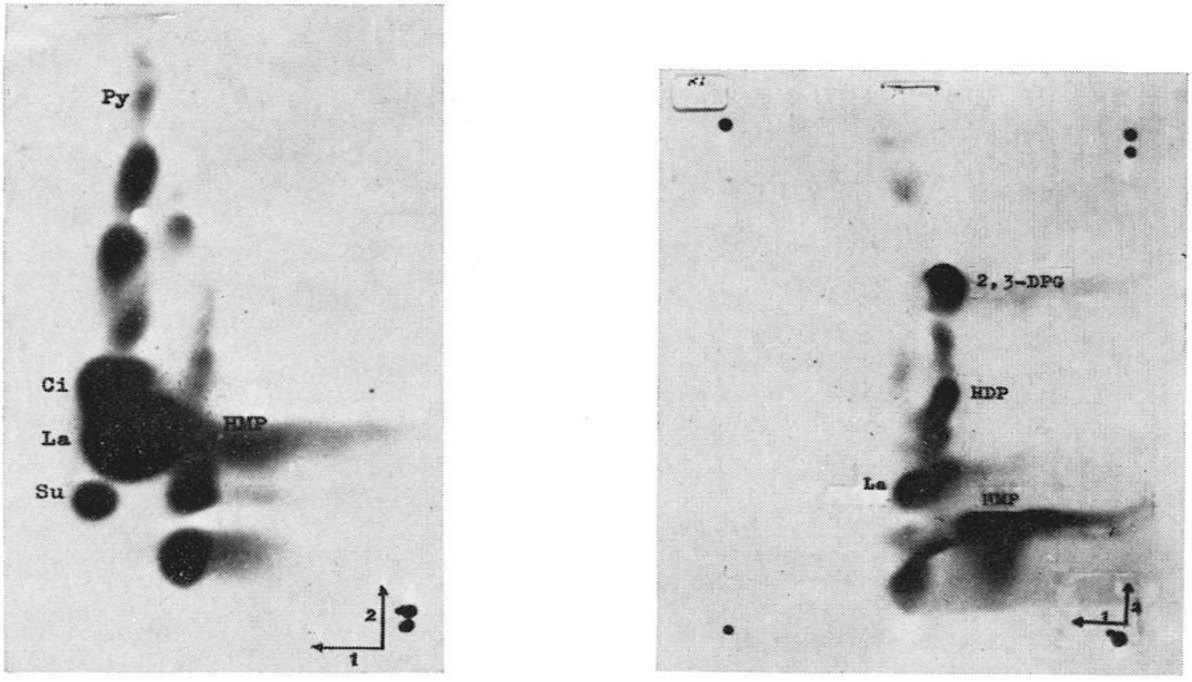


Fig. 3. Typical zone electrophoresis and isotachophoresis results. Electrolyte system: II + III and IV, according to Table I. To demonstrate the ability of the method to work with crude samples, some experiments were carried out on biological materials. (A) A muscle biopsy (about 20 mg, *Quadriceps femoris*) from man was incubated at 22° for 20 min with 4 mM (0.02 $\mu$Ci) [U-$^{14}$C]lactate in Krebs–Ringer bicarbonate solution[9] saturated with 5% $CO_2$ and 95% $O_2$. After the incubation the biopsy was pressed against the wedge-shaped filter-paper at the cathodic part of the strip. Isotachophoresis for the two-dimensional run was carried out with the biopsy acting as the sample. Several labelled substances were formed during the incubation. Carboxylic acids of the Krebs cycle such as citrate (Ci) and succinate (Su) and glycolytic metabolites such as pyruvate (Py) and hexosemonophosphates (HMP) were isolated, indicating oxidation of the lactate inside the mitochondria and at the same time synthesis of glycogen from the lactate. (B) Heparinized venous blood (0.2 mg/ml) from man was incubated at 22° for 20 min with 0.1 $\mu$moles (0.3 $\mu$Ci) of [U-$^{14}$C]glucose. Two-dimensional electrophoresis was run on the total amount of the incubation mixture (20 $\mu$l). In this case, only glycolytic metabolites such as hexosemonophosphates (HMP), hexosediphosphates (HDP), 2,3-diphosphoglycerate (2,3DPG) and lactate (La) could be proved to be present.

and enter the zone of ions of high mobility. The tendency to hold ions of the same mobility together in one zone means that the risk of tailing[7] is less in isotachophoresis than in zone electrophoresis, which means that the yield in isotachophoresis can be expected to be higher than in zone electrophoresis and that the risk of contamination is small. Theoretically, contamination should occur only in the boundaries between the different zones. The main part of the zones should be pure. In practice, 100 % purity of the main parts of the zones is attained only in favourable cases.

The possibility of applying crude enzymatic reaction mixtures to the isotachophoretic strip is advantageous for various reasons. By de-proteinisation and concentration of enzymatic reaction mixtures, fragile intermediates may be destroyed. Further, a substrate with a high mobility will move rapidly during the first stage of an isotachophoretic experiment out of the zone that contains enzymes, so that the consumption of substrate, say a fragile intermediate, by enzymatic reactions is efficiently stopped.

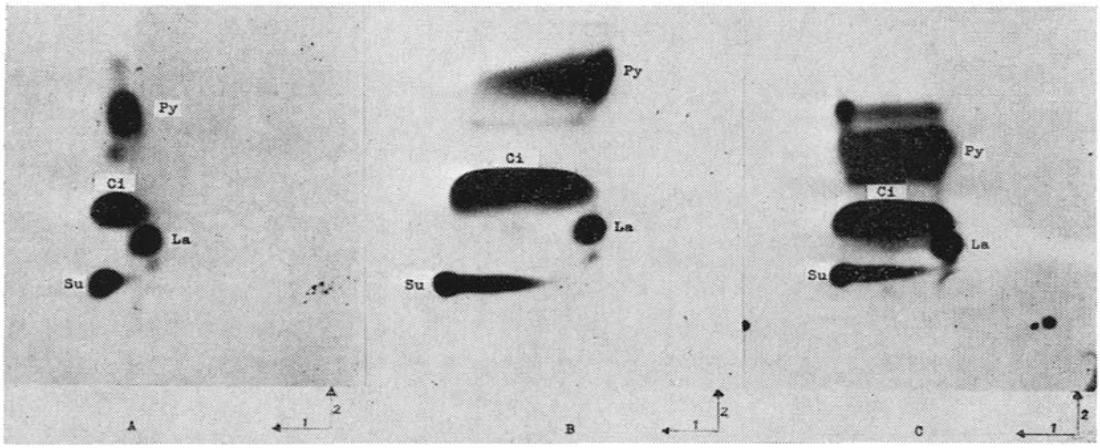


Fig. 4. Typical zone electrophoresis and isotachophoresis results. Electrolyte systems: I + III and IV (Figs. 4A and 4B), II + III and IV (Fig. 4C), according to Table I. During isotachophoresis, ionic species are arranged in consecutive zones. The lengths of the zones depend on the concentration of the leading ion and the amount of ionic species present in the system. The figure shows the result of experiments in which (A and B) the amount of citrate and (B and C) the concentration of the leading ion were varied. The system contained four glucose metabolites: citrate (Ci) of intermediate mobility, succinate (Su) of higher mobility and lactate (La) and pyruvate (Py) of lower mobility than citrate. In the first experiment (A), 0.4 $\mu$moles (0.08 mg) of citrate, and in the second (B), 1.9 $\mu$moles (0.4 mg) of citrate were added to the system. The distances between succinate and lactate were 14 mm and 42 mm, respectively. When the concentration of the leading ion was 0.4 *M* the distance was 34 mm (C). The results of these experiments are demonstrated graphically in Fig. 5.

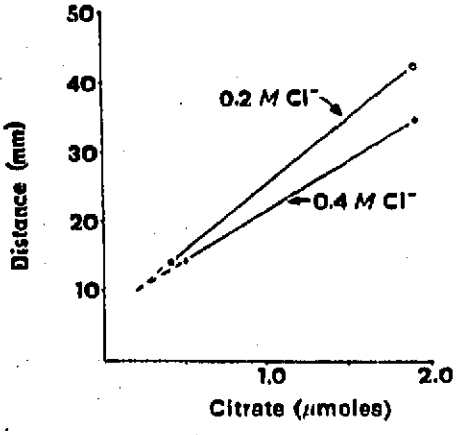


Fig. 5. The distance between the pyruvate and succinate zones as a function of the amount of citrate present in the separation mixture. Two different concentrations of leading ion were used (0.2 *M* and 0.4 *M* chloride).

As isotachophoresis can be performed, at least theoretically, at any pH and practically at many pH values, for instance at pH values around 7, it is possible to choose pH conditions to give optimum stability for labile ions. Also for the second dimension, different pH conditions can be used. One system, the EDTA system, has been found to work well in the neighbourhood of pH 7.

A very simple technique was used for the change from the first to the second dimension[1]. A portion of the isotachophoretic strip, containing the zones of interest, was cut out and placed on the thin layer of cellulose powder. When, for some reason, *e.g.*, incomplete separation during isotachophoresis, the separation in the first

dimension has to be re-run, the cut-out piece of the cellulose acetate strip, containing the zones of interest, was used as the "sample" in the second isotachophoretic separation. This re-running procedure is similar to that used by KENDALL AND CRITTENDEN[9] in attempts to separate isotopes 50 years ago.

In the experiments with whole blood, a zone of lipid compounds was formed, which sometimes caused distortion of the isotachopherogram. The cellulose acetate strip had a tendency to burn off near that zone. Also, in those experiments the simple re-running technique could be used. Here the zones of interest were transferred to the next isotachophoresis before the separation zone had reached the anodic edge of the cellulose acetate strip.

By the two-dimensional technique, the degree of completeness of the isotachophoretic separation can be controlled (Fig. 4). If two compounds of interest in the isotachophoretic separation are located in zones in immediate contact with each other, they may become separated in the second-dimension zone electrophoresis and be collected free from each other.

Figs. 4A and 4B also illustrate the "separation capacity" of the isotachophoretic system. In experiment A, the separation capacity is sufficient to develop zones that contain only one ionic species and the corresponding counter ion. In experiment B, in which the amount of citrate is five times as great as the amount used in A, the separation capacity has been exceeded. The main zone contains a number of substances. A five-times wider cellulose acetate strip would resolve mixture B (containing 0.4 mg of citrate) with the same degree of completeness as was attained in experiment A.

There are essential differences between a two-dimensional chromatogram and the two-dimensional separation described here. In a two-dimensional chromatogram, the position of a substance is, over a wide range, independent of the amount of the other compounds present in the separated mixture. In the system described here, the position of a substance is highly dependent on the amount of the other ions, as can be seen in Fig. 4. The concentration of a substance in a spot on a two-dimensional chromatogram is proportional to the concentration of the substance in the sample. In the system described here, the concentration in a spot is independent of the starting concentration. Because of the concentrating effect in isotachophoresis, there is also a good signal-to-background ratio for substances of low concentration in the sample, so that compounds that are present in only trace amounts can be detected.

The first-dimension isotachophoresis can be used not only to concentrate and separate ions but also to extract ions from tissues (Fig. 3). Although no proof of the completeness of the extraction has been obtained in this investigation, it seems likely that the procedure is efficient. If an essential part of the labelled compounds of high mobility was left in the tissue, one would expect a gradual release of labelled compounds, causing a radioactive tail. No such tail was found on the radioautograms.

## ABBREVIATIONS

The following abbreviations are used in this paper: α-k-gl = α-ketoglutarate; AMP, ADP and ATP = adenosine mono-, di- and triphosphate; β-OH-b = β-

hydroxybutyrate; Ci = citrate; DHAP = dihydroxyacetone phosphate; 1,3- and 2,3-DPG = 1,3- and 2,3-diphosphoglycerate; EDTA = ethylene diaminetetraacetic acid; E4P = erythrose-4-phosphate; F6P = fructose-6-phosphate; F1P = fructose-1-phosphate; FDP = fructose-1,6-diphosphate; Fu = fumarate; GAP = glyceraldehyde-3-phosphate; G3P = glycerol-3-phosphate; G6P = glucose-6-phosphate; HMP and HDP = hexose mono- and diphosphate; H6P = hexose-6-phosphate; La = lactate; Ma = malate; NADP = nicotine adenine dinucleotide phosphate; ODP = octulose-1,8-diphosphate; P5P = pentose-5-phosphate; PEP = phosphoenolpyruvate; 2- and 3PGA = 2- and 3-phosphoglycerate; 6 PG= 6-phosphogluconate; Pi = inorganic orthophosphate; Py = pyruvate; RuDP = ribulose-1,5-phosphate; R5P = ribose-5-phosphate; Ru5P = ribulose-5-phosphate; SDP = sedoheptulose-1,7-diphosphate; S7P = sedoheptulose-7-phosphate; Su = succinate; Tris = tris (hydroxymethyl) aminomethane.

## REFERENCES

1 A. VESTERMARK AND B. SJÖDIN, *J. Chromatogr.*, 71 (1972) 588.
2 E. NEGELEIN AND H. BRÖMEL, *Biochem. Z.*, 303 (1940) 132.
3 C. E. BALLOU, H. O. L. FISHER AND P. L. MACDONALD, *J. Amer. Chem. Soc.*, 77 (1955) 5967.
4 Z. DISCHE, *Ann. N.Y. Acad. Sci.*, 75 (1958) 129.
5 B. AXELROD AND R. JANG, *J. Biol. Chem.*, 209 (1954) 847.
6 H. E. WACH AND D. M. MORGAN, *Biol. J.*, 56 (1954) 41.
7 F. KOHLRAUSCH, *Ann. Phys. (Leipzig)*, 62 (1897) 209.
8 H. HAGLUND, *Sci. Tools*, 17 (1970) 2.
9 J. KENDALL AND E. D. CRITTENDEN, *Proc. Nat. Acad. Sci.*, 9 (1923) 75.
10 H. F. DE LUCA AND P. P. COHEN, in W. W. UMBREIT, R. H. BURIS AND J. F. STAUFFER (Editors), *Manometric Techniques*, Burgess Publishing Co., 4th ed., 1964, p. 132.