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QUANTITATIVE DETERMINATION OF GLUCOSE METABOLITES SEPARATED BY ISOTACHOPHORESIS IN TWO-DIMENSIONAL COMBINATION WITH ZONE ELECTROPHORESIS

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SUMMARY

The experimental conditions used for the separation of glucose metabolites by isotachophoresis in two-dimensional combination with zone electrophoresis were arranged so as to give good quantitative yields.

INTRODUCTION

In preceding papers[1,2] we have described how isotachophoresis alone or combined with zone electrophoresis has been used to separate glucose metabolites from biological incubation mixtures. In this paper, the possibility of using the same methods for quantitative analysis is described.

MATERIALS

Unlabelled lactate, pyruvate, NAD and NADH and the enzyme lactate dehydrogenase (LDH) were obtained from Boehringer & Söhne GmbH, G.F.R. Sodium [1-^{14}C]lactate (25.6 mCi/mmole), sodium [1-^{14}C]pyruvate (23.8 mCi/mmole) and [2,3-^{14}C]succinate (16.7 mCi/mmole) were obtained from the Radiochemical Centre, Amersham, Great Britain.

METHODS

Electrophoresis

The separation technique described earlier[1] was used. The electrolytes used for the two-dimensional electrophoresis are summarized in Table I.

Determination of radioactivity

The radioactivity of ^{14}C-labelled substances was measured in a Packard 3375 Tri-carb scintillator counter. The samples were counted in glass scintillator vials. The scintillation solution consisted of 0.5 % 2,5-diphenyloxazole (PPO), 0.03 % 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (POPOP) dissolved in 86.7 % toluene and 13.3 % methanol. The total volume of solution in the vial was 15 ml.

TABLE I

ELECTROLYTES USED FOR TWO-DIMENSIONAL ELECTROPHORESIS

Technique	*Buffer system*	*Concentration (M)*	*pH*	*Cathode vessel*	*Anode vessel*	*Strip or plate*	*Direction*
Isotachophoresis	Tris–HCl	0.4	7.2		+	+	1
	Tris–ascorbate	0.2	7.2	+			1
Zone electrophoresis	Ammonium formate	0.2	3.75	+	+	+	2

To investigate the optimum counting conditions, [2,3-^{14}C]succinate, [1-^{14}C]-lactate and [1-^{14}C]pyruvate were used. The solutions of the three labelled compounds were adjusted to pH 6 with Tris–hydroxide. Then 20-μl amounts of the solutions were added to counting vials containing scintillation solution and different amounts of water, cellulose powder, unlabelled carrier and methanol were also added.

The conditions used in the different experiments are summarized in Table II.

TABLE II

EXPERIMENTAL CONDITIONS

Experiment	*Water (μl)*	*Cellulose powder (mg)*	*Methanol (%)*	*Carrier (M)*
A	0–80	—	13.3	—
B	20	0–80	13.3	—
C	20	25	13.3	0–1
D (a)	20	25	0–20	—
D (b)	60	25	0–20	—
D (c)	60	60	0–20	—

Drying of the cellulose thin layers

In four series of experiments, the yield of radioactivity was studied. In three of the experiments, ^{14}C-labelled succinate, lactate and pyruvate dissolved in 0.2 *M* ammonium formate buffer of pH 4 were used. In one series, a pyruvate solution adjusted to pH 6 with Tris–hydroxide was used. The solutions contained 0.5 % of the blue dye indigo tetrasulphonate. Aliquots of 20 μl of the two solutions were applied to a thin layer of dry cellulose. The thin layer was dried in an oven for 0–120 sec at 90 – 110 °. One spot containing labelled pyruvate of pH 6 was dried for 120 sec at 130 °.

The cellulose powder in the blue-coloured areas was scraped out and transferred to vials containing scintillator solutions.

The yield of chemical amounts after drying was studied in two series of experiments with unlabelled pyruvate and lactate. Amounts of 20 μl of solutions of the two acids in 0.2 *M* ammonium formate buffer of pH 4 containing 0.5 % indigo tetrasulphonate were applied to thin layers of cellulose. The thin layers were dried at an

oven temperature of 90–110°, as described earlier. During the drying, a thermometer was held in contact with the upper (cellulose) side of the thin layer.

After drying, the cellulose powder in the blue-coloured areas was transferred to test-tubes containing 1.05 ml of water. The lactate concentration was determined in 200-μl amounts of suspension according to the method of SCHOLZ *et al.*[3], and the pyruvate concentration in 500-μl amounts of suspension by the method of BÜCHER *et al.*[4].

Determination of recovery of radioactivity from succinate after separation from blood

Succinate was used to determine the recovery of the radioactivity (c.p.m.) after separation from blood by two-dimensional electrophoresis.

From a stock solution containing pure [2,3-^{14}C]succinate (3000 d.p.m./μl), the following solutions were made:

(A) Blood and stock solution, 1:1.

(B) Distilled water and stock-solution, 1:1.

Two-dimensional electrophoresis was carried out on 50 μl of solution A with systems II and III (Table I). The radioactivity of the scraped-out spot containing succinate was also measured in 50 μl of solution B.

Determination of recovery of the chemical amounts of lactate and pyruvate after separation

Two-dimensional electrophoresis was carried out on 50-μl samples of the following solutions: (1) Na lactate + Na [1-^{14}C]lactate, 7 m*M*, 10,000 d.p.m., 0.01% indigo tetrasulphonate; (2) Na pyruvate + Na [1-^{14}C]pyruvate, 0.5 m*M*, 10,000 d.p.m., 0.01% indigo tetrasulphonate.

The plates were dried at 90–110° for 80 sec. Lactate and pyruvate were localized autoradiographically. The areas containing the acids were scraped out and transferred to two test-tubes, A_1 and A_2, containing 1.05 ml of water. Into two other test-tubes, B_1 and B_2, containing 1 ml of water, 50 μl of solutions 1 and 2, respectively, were transferred by pipette. The quantitative determinations were carried out as described under *Drying of the cellulose thin layers*.

RESULTS

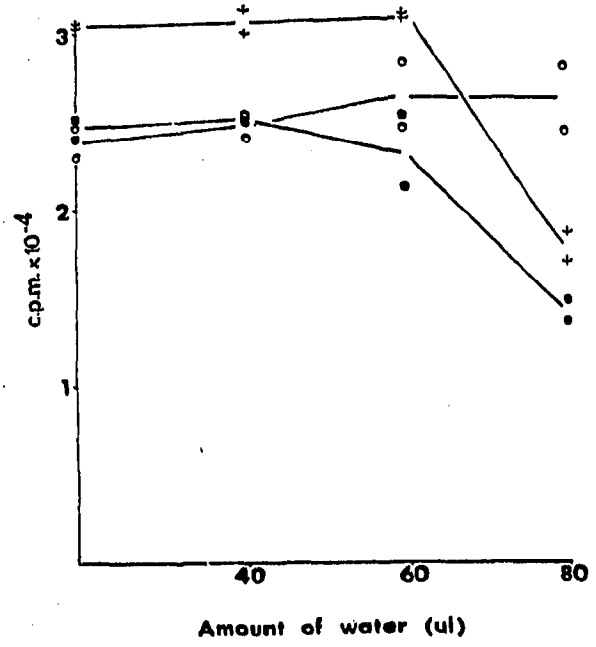
The results of the determination of the radioactivity under different conditions are summarized in Figs. 1–4. From the figures, it can be seen that it is possible to count the ^{14}C-labelled acids with good results in the presence of cellulose powder containing water.

From Figs. 5 and 6 it can be seen that it is necessary to dry the cellulose thin-layer plates at a pH not lower than 6 when the drying temperature is 90–110°, in order to avoid losses of pyruvate. When the drying is performed at 130°, losses occur at pH 6.

The same conclusion can be drawn from the determination of the yields of chemical amounts (Figs. 7 and 8).

In Table III is shown the result of the determination of the radioactivity from succinate after its separation from blood. The recovery is good.

In Table IV is shown the result of the determination of the recovery of the chemical amounts of lactate and pyruvate after their two-dimensional separation.



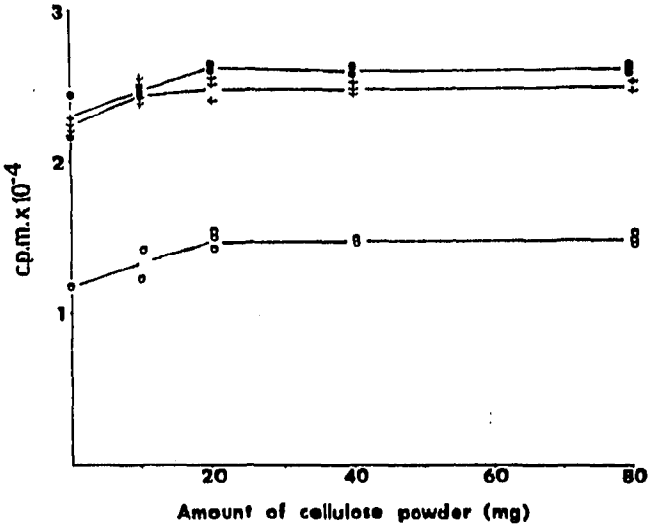
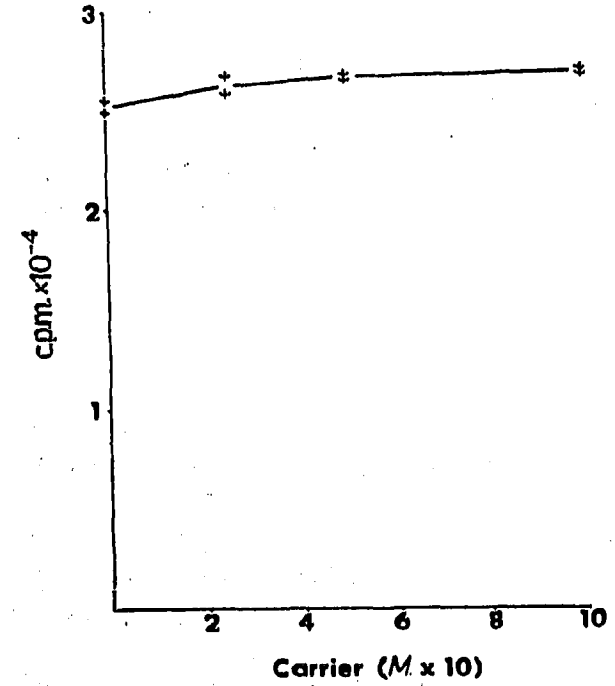


Fig. 1. Determination of radioactivity. Experimental conditions: Table II, experiment A. Symbols: +, [1-^{14}C]pyruvate; ●, [1-^{14}C]lactate; ○, [2,3-^{14}C]succinate. The figure shows that the radioactivity of the ^{14}C-labelled compounds could be measured with reliable results in up to 40 μl of water under these conditions.

Fig. 2. Determination of radioactivity. Experimental conditions: Table II, experiment B. Symbols: +, [1-^{14}C]pyruvate; ●, [1-^{14}C]lactate; ○, [2,3-^{14}C]succinate. This experiment shows that the efficiency of the counting increased in the presence of cellulose powder.



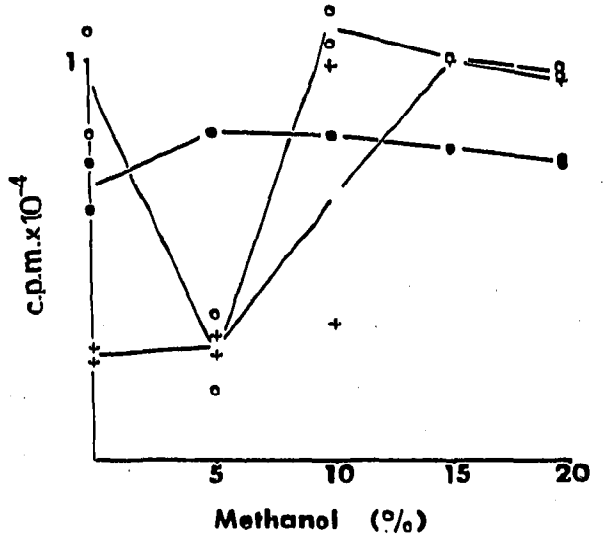


Fig. 3. Determination of radioactivity. Experimental conditions: Table II, experiment C, with [1-^{14}C]pyruvate. The efficiency of the counting increased slightly in the presence of carrier.

Fig. 4. Determination of radioactivity. Experimental conditions: Table II, experiment D. Symbols: ●, samples containing the mixture described in experiment D (a); +, experiment D (b); ○, experiment D (c). The methanol concentration could, as the figure shows, be kept low when the samples contained small amounts of water. At high contents of water, the amount of cellulose powder had to be kept high.

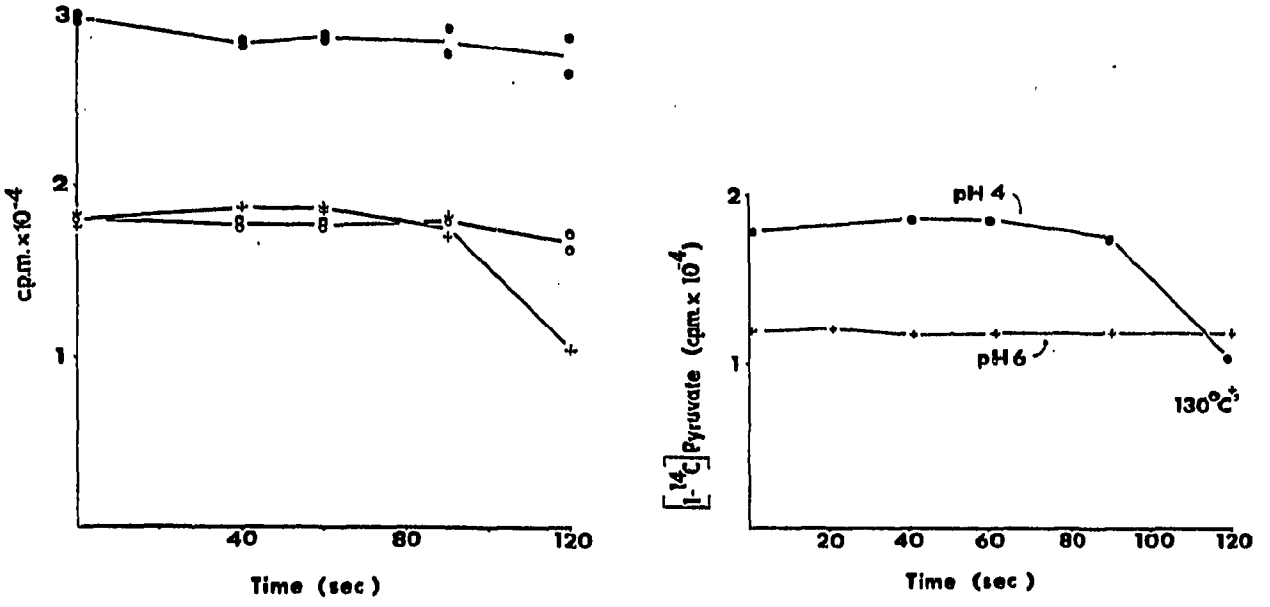


Fig. 5. Determination of radioactivity. Experimental conditions as described under *Drying of the cellulose thin layers*. The pH was adjusted to 4. Symbols: +, [1-^{14}C]pyruvate; ●, [1-^{14}C]-lactate; ○, [1-^{14}C]succinate. Pyruvate at pH 4 started to evaporate after 80 sec when the water had disappeared from the thin layer.

Fig. 6. Determination of radioactivity. Experimental conditions as in Fig. 5. Symbols: +, [1-^{14}C]pyruvate at pH 6; ●, [1-^{14}C]pyruvate at pH 4. At pH 6, the counts of [1-^{14}C]pyruvate did not decrease when the plate was dried at 90–100 ° for 120 sec. At a temperature of 130 °, 25% disappeared.

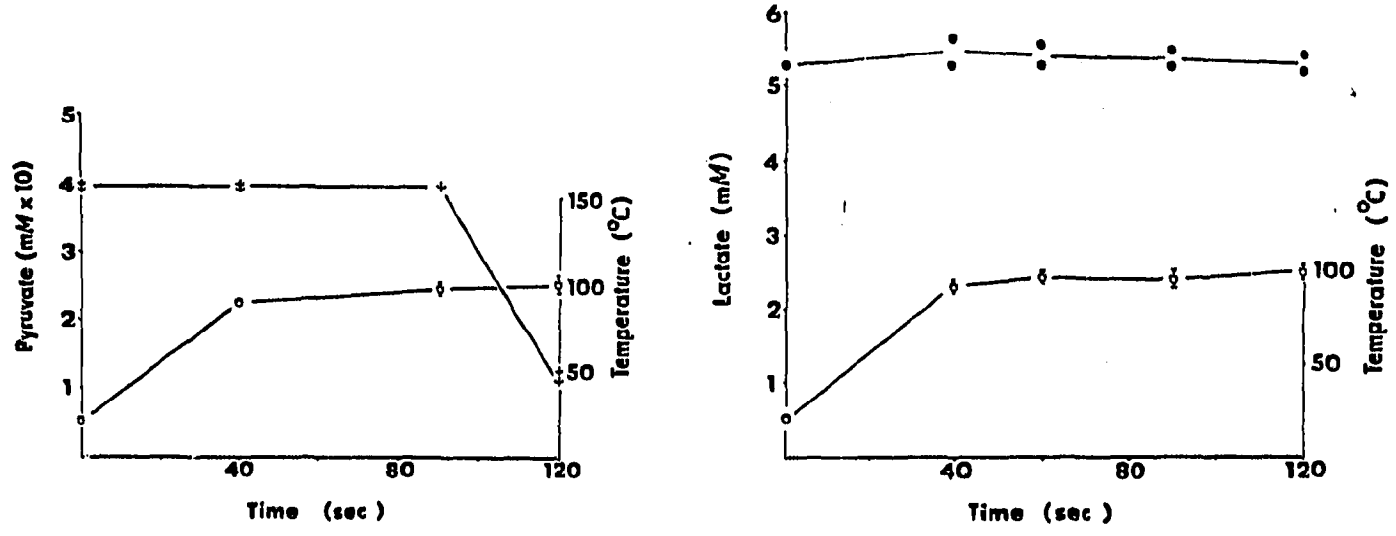


Fig. 7. Determination of radioactivity. Experimental conditions as in Fig. 5. Symbols: +, [1-^{14}C]pyruvate; ○, temperature. The results are principally the same as shown in Fig. 5.

Fig. 8. Determination of radioactivity. Experimental conditions as in Fig. 5. Symbols: ●, [1-^{14}C]lactate; ○, temperature. The results are principally the same as shown in Fig. 5.

The ^{14}C-labelled pyruvate gave rise to three spots, behaving enzymatically as pyruvate (Fig. 9). The yield of pyruvate was lower than that of lactate, probably because of loss of pyruvate during drying.

TABLE III

DETERMINATION OF RECOVERY OF NUMBER OF COUNTS FROM SUCCINATE

Solution	*Radioactivity (c.p.m.)*	*Number of samples*	*Recovery (%)*
A	66,133 ± 4384	8	99 ± 6
B	67,019 ± 3410	5	

TABLE IV

DETERMINATION OF RECOVERY OF CHEMICAL AMOUNTS OF LACTATE AND PYRUVATE

Substance	*Solution (1)*		*Solution (2)*		
	Concentration (mM)	*Number of samples*	*Concentration (mM)*	*Number of samples*	*Recovery (%)*
Lactate	6.3 ± 0.3	7	6.8 ± 0.1	4	93 ± 8
Pyruvate	0.40 ± 0.02	10	0.49 ± 0.04	8	81 ± 5

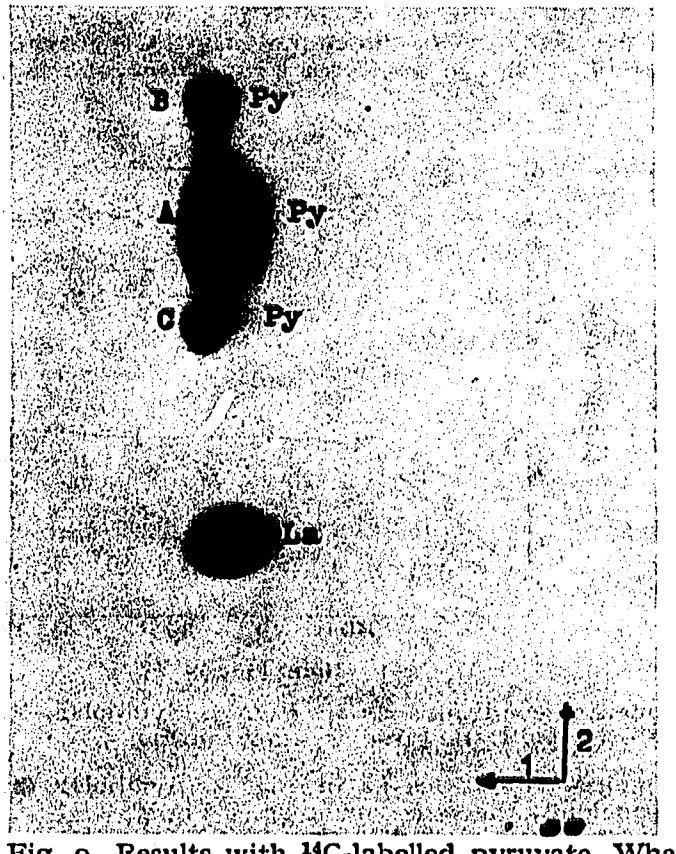


Fig. 9. Results with ^{14}C-labelled pyruvate. When venous blood from man was incubated with [1-^{14}C]pyruvate, the pyruvate was converted to lactate. The incubation time was 30 sec at 22 °. As illustrated, pyruvate was divided into three spots, all of which contained substances that were reduced to lactate in the presence of LDH and NADH. The main spot A is probably pyruvate but the other two, B and C, might be polymers of pyruvate, which are easily formed at low pH[5].

DISCUSSION

From the results, it can be seen that a two-dimensional combination with isotachophoresis can be used for quantitative purposes. The radioactivity of ^{14}C-labelled compounds can be determined directly on the scraped-out powder from the dried thin layers. The thin layers can be dried, within limits, in a crude and simple manner without loss of ^{14}C-labelled substances. Of course, there are temperature-sensitive substances for which milder drying procedures must be used. When such compounds have been separated, autoradiography can be carried out before drying in a freeze-box at −20°. Afterwards the thin layer can, if necessary, be freeze-dried.

The yield is high, so that the risk of tailing is low and also the risk of contamination is low.

The yield determined by enzymatic methods was good, which is important because it shows that the compounds on the dried cellulose thin layer are intact after separation and drying. This is favourable when the method is used, for instance, for the small-scale preparation of intermediates synthesized by enzymatic methods.

The two-dimensional combination of isotachophoresis with zone electrophoresis can be used for the concentration, separation and quantitative determination of glucose metabolites.

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