

Le fait que la phosphorescence ne se produit que si la substance est adsorbée par un support est peut-être explicable par une augmentation de stabilité produite par une interaction physicochimique avec la cellulose.

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### **Paper chromatographic separation of adenine metabolites: purine bases, nucleosides, and nucleotides**

During work on the fate of 8-<sup>14</sup>C-adenine in the isolated rat diaphragm, it was necessary to obtain a paper chromatographic separation of a mixture of numerous substances of different chemical types (purine bases, nucleosides, and nucleotides), with cleanly separated spots so as to permit both their identification and the measurement of their radioactivity with a chromatoscanner.

It was not possible to obtain this by using one-dimensional chromatography with the solvents reported in the literature<sup>1-6</sup>. In addition the  $R_F$  values of only some of the substances of interest in the aforementioned research were reported in the literature (and at times the values of different workers did not agree).

In the present work the  $R_F$  values have been determined with reference substances dissolved in water or in the saline solution in which the samples to be analyzed were chromatographed. Further, the authors preferred to couple solvent systems already described in the literature for one-dimensional chromatograms in order to obtain a two-dimensional chromatogram which gives greater resolution.

#### *Experimental*

*Paper chromatography.* Descending development on Whatman No. 1 paper in a tank kept in a thermostated room (temperature 18° + 2°) was used. The size of the one-dimensional chromatogram was 53 × 31 cm; the two-dimensional chromatogram was 46 × 41 cm. Samples were placed at 8.5 cm from the edge of the papers.

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Before developing, the atmosphere of the chromatographic tank was saturated by covering the base of the tank with a small quantity of the solvent.

In cases where the liquid was allowed to run off the end of the paper, the sheet was cut with pinking shears to ensure that the solvent would descend uniformly.

The reference substances used (analytically pure grade) were dissolved in water, or in dilute bases at concentrations of about 2 mg/ml. Aliquots of 5  $\mu$ l were placed on the paper with a micrometer syringe. An equal volume was added to the sample to be analyzed. Both volumes were deposited in small drops and the evaporation accelerated by heating the lower part of the paper with a current of hot air.

*Detection of the substances.* All the reference substances were detected by viewing the absorption at 253.7 nm by means of a low-pressure mercury vapor lamp (with a Corning 9863 filter):

### Results

After an examination of the data in the literature, solvents which might be

TABLE I

$R_{Hy}$ <sup>a</sup> VALUES OF PURINE, PYRIMIDINE, PURINE NUCLEOSIDES AND PURINE NUCLEOTIDES<sup>b</sup>

Compounds	Solvents <sup>c</sup>					
	S <sub>1</sub>	S <sub>2</sub>	S <sub>3</sub>	S <sub>4</sub>	S <sub>5</sub>	S <sub>6</sub>
Adenine	1.20	1.06	0.85	1.02	1.34	0.67
Guanine	0.90	0.58	0.56	0.72 dec	0.63	0.71
Cytosine	0.93	1.14	0.74	1.05	1.35	1.25
Uracil	1.07	1.09	1.41	1.11	1.30	1.29
Hypoxanthine	1.00	1.00	1.00	1.00	1.00	1.00
Xanthine	0.87	0.73	0.75	0.88 dec	0.61	0.84
Uric acid	0.66	0.39	0.43	0.62	0.36	0.67
Adenosine	1.06	1.19	0.57	1.00	1.40	0.89
Guanosine	0.80	0.73	0.39	0.89	0.63	1.11
Inosine	0.82	0.93	0.50	0.96	0.70	1.28
Xanthosine	0.77	0.77	0.38	0.83	0.48	1.17
AMP	0.51	0.32	0.07	0.70	0.12	1.23
ADP	0.27	0.23	0.00	0.44	0.09	1.38
ATP	0.20	0.19	0.00	0.30	0.07	1.45
GMP	0.39	0.14	0.06	0.55	0.05	1.39
IMP	0.43	0.20	0.07	0.67	0.06	1.56

<sup>a</sup>  $R_{Hy} = R_F$  relative to hypoxanthine.

<sup>b</sup> All the values (the average of five determinations) were obtained from one-dimensional descending chromatograms on Whatman No. 1 paper at  $18 \pm 2^\circ$ . The partial decomposition in a solvent is indicated by dec.

<sup>c</sup> Solvent systems used:

S<sub>1</sub>: *n*-butanol-acetic acid-water (40:11:25 by vol.).<sup>1,2</sup> Hypoxanthine runs about 30 cm in 40 h of development with overflow. Its  $R_F$  is 0.46.

S<sub>2</sub>: *n*-propanol-ammonium hydroxide ( $d = 0.90$ )-water, (3:1:1 by vol.).<sup>3</sup> Hypoxanthine runs 30 cm in 40 h of development with overflow. Its  $R_F$  is 0.53.

S<sub>3</sub>: *n*-butanol-99% formic acid-water (77:10:13 by vol.).<sup>3</sup> Hypoxanthine runs 40 cm in 10 h of development with overflow. Its  $R_F$  is 0.27.

S<sub>4</sub>: 95% ethanol-*M* ammonium acetate (pH 3.8) (75:30 by vol.).<sup>4</sup> Hypoxanthine runs 32 cm in 27 h of development with overflow. Its  $R_F$  is 0.54.

S<sub>5</sub>: *n*-butanol-diethyl glycol-water (4:1:1 by vol.) ( $\text{NH}_3$  atmosphere).<sup>5</sup> Hypoxanthine runs 35 cm in 70 h of development with overflow. Its  $R_F$  is 0.31.

S<sub>6</sub>: 5% (w/v)  $\text{Na}_2\text{HPO}_4$  in isoamyl alcohol (100:50 by vol.).<sup>6</sup> Hypoxanthine runs 25 cm in 10 h of development with overflow. Its  $R_F$  is 0.60.

suitable for the separation of the mixtures of substances of the types indicated above were selected. These solvents were tried and the results are reported in Table I. To obtain a greater separation, the development of many of the solvents was prolonged by allowing the solvent to run off the paper. Hypoxanthine was chosen instead of the solvent front for reference and in Table I the  $R_F$ 's relative to hypoxanthine are reported (indicated by  $R_{Hy}$ ).

Two-dimensional development with pairs of some of the solvent systems described in Table I clearly resulted in improved separation.

*Example.* As an example of the utilization of the data of Table I, the case of the separation and identification of the metabolites contained in the incubation medium and in the aqueous alcoholic extract of a rat diaphragm incubated with 8- $^{14}$ C-adenine is described. The biochemical particulars of this work will be reported elsewhere<sup>7</sup>.

The samples mentioned were chromatographed with the solvents indicated in Table I and autoradiographs of the chromatograms obtained are shown in Fig. 1. In the case of the extracts, up to eight spots were observed, in the case of the medium up to five. The  $R_{Hy}$  of these spots are reported in Table II. By comparing the  $R_{Hy}$  in the two tables and allowing for a variation up to  $\pm 0.1$ , a preliminary identification of the substances producing the spots in Table II may be made.

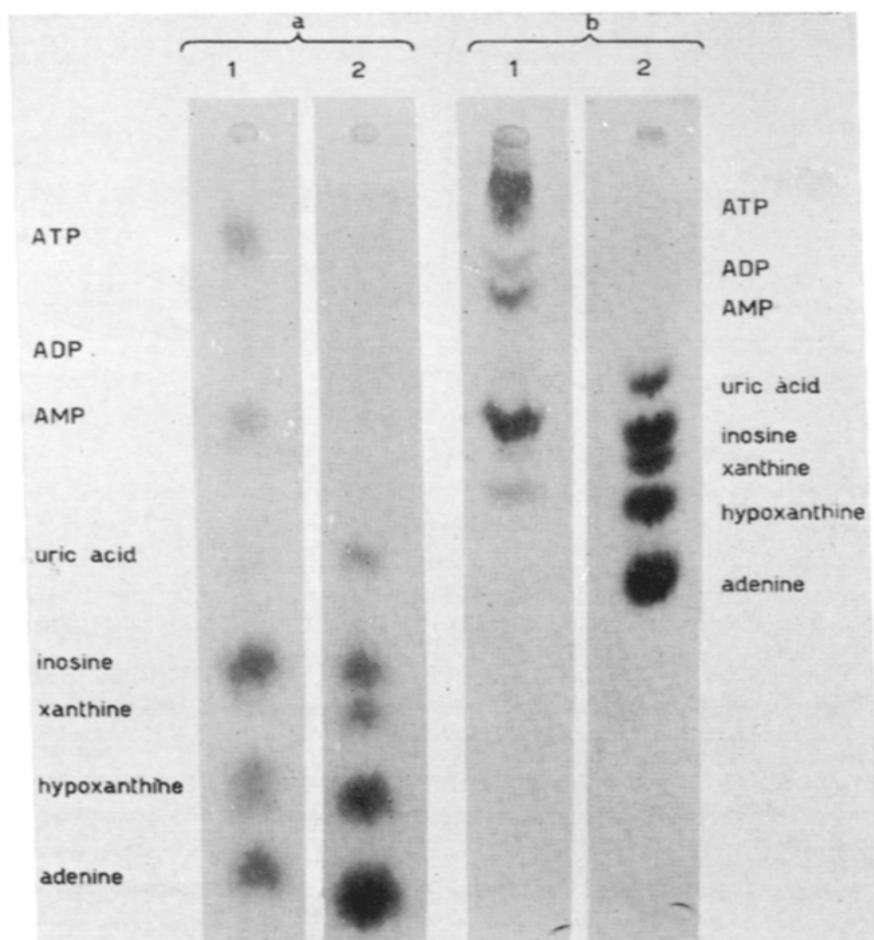


Fig. 1. Autoradiograph of one-dimensional chromatograms (developed in solvent  $S_1$ ), of the alcoholic extract (1), and of the medium after incubation (2) of rat diaphragm incubated with 8- $^{14}$ C-adenine; (a) with and (b) without running off the paper.

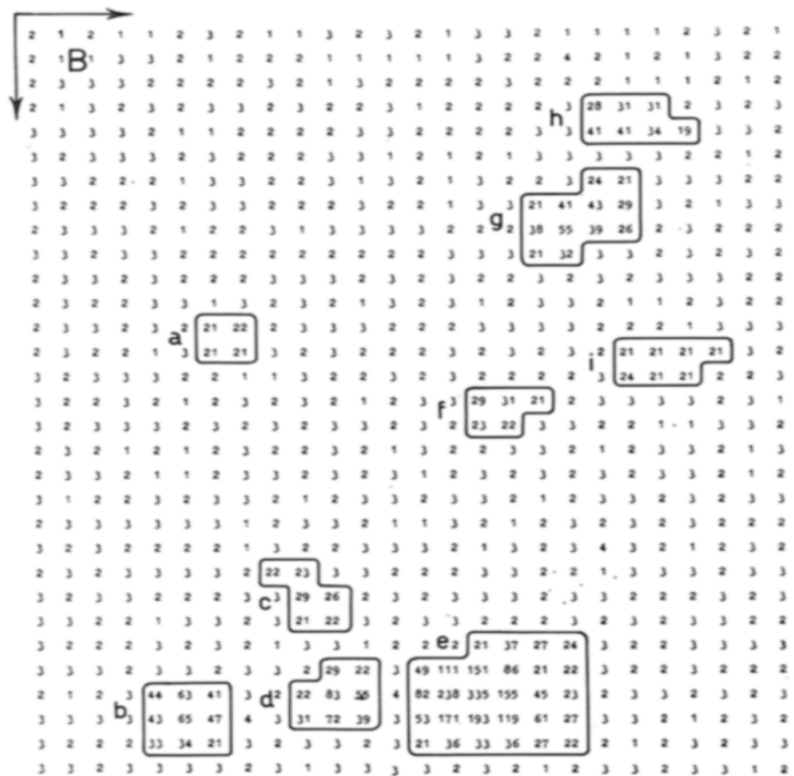
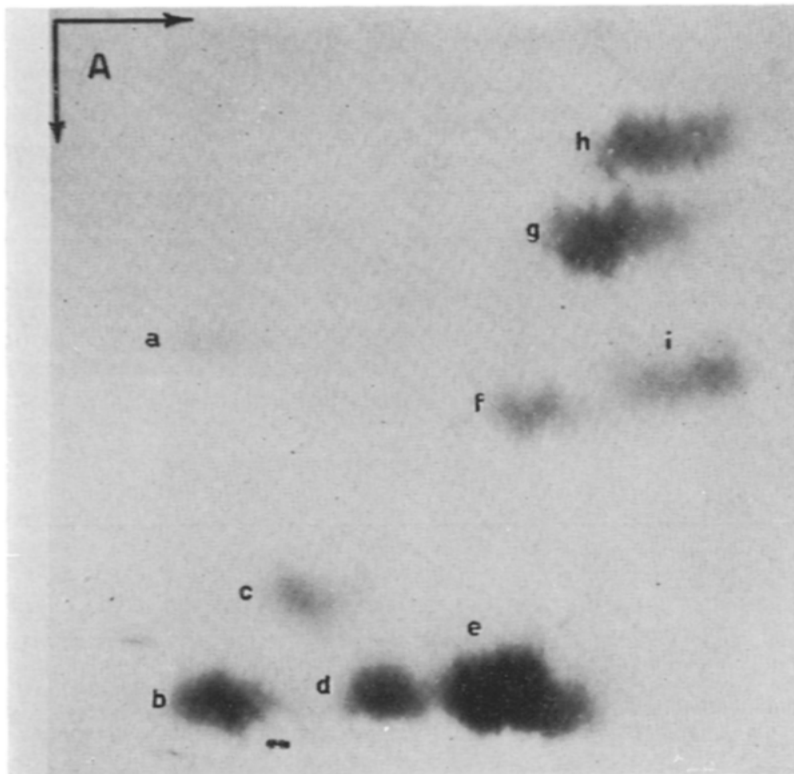


Fig. 2. Autoradiograph (A) and numerical map of the activity (B) of a two-dimensional radiochromatogram of an aqueous alcoholic extract of rat diaphragm incubated with 8-<sup>14</sup>C-adenine (the chromatogram was developed: horizontally in solvent S<sub>6</sub> (8 h), vertically in solvent S<sub>4</sub> (30 h, solvent allowed to run off paper)). a = Uric acid; b = adenine; c = xanthine; d = hypoxanthine; e = inosine; f = AMP; g = ADP; h = ATP; i = IMP.

TABLE II

$R_{HY}$  VALUES OF THE RADIOACTIVE SPOTS OF THE CHROMATOGRAM OF THE EXTRACTS AND INCUBATION MEDIUM WHICH CONTAIN SUBSTANCES LISTED IN TABLE I

The values in parentheses refer to the incubation medium.

<i>Solvents</i>					
$S_1$	$S_2$	$S_3$	$S_4$	$S_5$	$S_6$
0.21	0.19	(0.37)	0.25	0.07	0.65 (0.66)
0.30	0.24	(0.48)	0.39	0.14	0.83 (0.83)
0.47	0.37	(0.75)	0.65 (0.62)	0.36 (0.38)	1.00 (1.00)
0.66 (0.65)	0.49 (0.44)	(0.93)	0.84 (0.82)	0.59 (0.61)	1.32 (1.27)
0.82 (0.80)	0.72 (0.67)	(1.00)	1.00 (1.00)	0.79 (0.81)	
0.88 (0.87)	0.93 (0.92)			1.00 (1.00)	
1.00 (1.00)	1.00 (1.00)			1.28 (1.28)	
1.18 (1.17)	1.12 (1.17)				

In order to make a comparison between substances running under identical conditions, reference substances were added to the mixtures and chromatographed. The values obtained for the  $R_{HY}$  are reported in Table III. Since in this case both the known and unknown substances were chromatographed together, those values in which the  $R_{HY}$  does not deviate more than 0.05 can be considered identical.

In order to exclude the possibility that some of the radioactive spots consist of two substances (of which one, possibly, might not be included in Table I), the radioactivity of various spots was measured in chromatograms developed in various solvents. The radioactivity was measured by exploring the radiochromatogram with a chromatoscanner with a square window one centimeter wide, made in this Institute<sup>8</sup>. The results of comparing the radioactivity of the spots in various

TABLE III

$R_{HY}$  VALUES OF A NUMBER OF THE SUBSTANCES OF TABLE I, CHROMATOGRAPHED TOGETHER WITH THE SAMPLES ANALYZED

The values in parentheses refer to the substances added to incubation medium.

<i>Compounds</i>	<i>Solvents</i>					
	$S_1$	$S_2$	$S_3$	$S_4$	$S_5$	$S_6$
Adenine	1.18 (1.17)	1.12 (1.17)	(0.93)	1.00 (1.01)	1.28 (1.28)	0.66 (0.66)
Guanine	dec dec	0.57 (0.58)	dec	0.72 (0.73)	0.67 (0.69)	0.81 (0.81)
Hypoxanthine	1.00 (1.00)	1.00 (1.00)	(1.00)	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)
Xanthine	0.88 (0.86)	0.72 (0.67)	dec	0.84 (0.82)	0.59 (0.61)	0.83 (0.83)
Uric acid	0.65 (0.66)	0.48 (0.45)	(0.37)	0.65 (0.62)	0.36 (0.38)	0.67 (0.66)
Adenosine	1.05 (1.03)	1.27 (1.27)	(0.61)	1.00 (1.00)		0.92 (0.92)
Guanosine	0.78 (0.77)	0.64 (0.64)	(0.33)	0.88 (0.86)	0.70 (0.71)	1.10 (1.10)
Inosine	0.82 (0.79)	0.93 (0.93)	(0.48)	0.99 (0.99)	0.79 (0.81)	1.29 (1.28)
AMP	0.45	0.37		0.67	0.14	1.27
ADP	0.30	0.24		0.39	0.08	1.34
ATP	0.21	0.19		0.25	0.06	1.37
GMP	0.39	0.14		0.51	0.06	1.36
IMP	0.37	0.23		0.62	0.07	1.50

chromatograms show that, under these conditions, it was not possible to deduce the presence of more than eight substances.

In the samples investigated, the following metabolites of adenine were found by the aforementioned methods:

In the medium after incubation: adenine (not metabolized), hypoxanthine, xanthine, inosine, uric acid.

In the extract (in addition to the aforementioned metabolites): AMP, ADP, ATP.

In order to obtain a better separation and to make it possible to perform the above mentioned measurements with the chromatoscanner, some two-dimensional developments coupling some of the solvents in Table I were performed; for example, the pairs  $S_6-S_4$ ,  $S_6-S_1$ ,  $S_2-S_1$  were used. As a result of such two-dimensional chromatograms another spot (identified as IMP) was resolved. This spot has a weak activity, and so it was not possible to detect it when comparing the activity of the spots in various one-dimensional developments.

An autoradiograph of a two-dimensional development with a pair of solvent systems particularly suitable for the mixtures in question is shown in Fig. 2. Because of the wide separation of the spots, a numerical map of the activities could be obtained with the chromatoscanner. This is also shown in Fig. 2.

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