THE SEPARATION OF NUCLEOTIDES FROM ACID-SOLUBLE TISSUE EXTRACTS BY HIGH-VOLTAGE PAPER ELECTROPHORESIS

H. M. KLOUWEN
with the technical assistance of H. WEIFFENBACH
Radiobiological Institute of the Organization for Health Research T.N.O., Rijswijk, Z.H. (The Netherlands)

(Received April 24th, 1961)

INTRODUCTION

The methods used for the separation of nucleotides are based either on ion exchange chromatography, paper electrophoresis or on a combination of these separation principles[1–5]. In general these methods are time-consuming and the nucleotides are often exposed to low pH values for several hours[1]. These conditions enhance decomposition of di- and triphosphate nucleotides. TURBA *et al.*[6] described the principle of separation of nucleotides by high-voltage paper electrophoresis and applied it among others, to the separation of nucleotides from yeast. They did not, however, identify the nucleotide fractions after separation.

In this communication the application of high-voltage paper electrophoresis for the separation of nucleotides from acid-soluble animal tissue extracts will be described.

Identification of the nucleotides was carried out by comparison of the relative mobilities of the nucleotides with those of reference compounds and furthermore by paper chromatographic analysis of the purines and pyrimidines obtained from them.

METHODS AND MATERIALS

Acid-soluble tissue extracts were prepared by homogenization in cold 2% $HClO_4$ immediately after excision of the tissues. The extracts were neutralized with 6 *N* KOH and, after centrifugation in order to remove $KClO_4$, lyophilized and taken up in a small amount of distilled water (100 μl water per 100 mg starting material). In a number of experiments the extracts were purified after neutralization by a charcoal column treatment[7] and eluted with 50% ethanol containing 1% NH_4OH. The nucleotide-containing solution was concentrated under reduced pressure and subsequently by lyophilization and taken up in a small volume of distilled water as described above. The preparation and purification of the acid-soluble extracts were carried out in the cold (0–3°).

Electrophoresis was carried out under toluene, as described by RYLE *et al.*[8] with

some modifications. A pyridine–acetate buffer pH 3.6 of 10 ml pyridine and 100 ml glacial acetic acid diluted with water to 1000 ml was used. The electrophoresis was performed on Whatman No. 1 paper, pretreated by washing with 1 *N* HCl and with water. Amounts of the acid-soluble extracts isolated from 100–200 mg tissue were applied in a narrow stripe to paper strips (45 cm long, 12 cm wide) over a distance of 8–9 cm. 2000 V was applied for 1 hour. After electrophoresis the strips were dried for 12 hours in air.

Nucleotides were localized by contact photography in ultraviolet light (U.V.) as described by MARKHAM AND SMITH[9]. Pyridine, which interferes strongly with the U.V.-absorption of nucleotides, can be removed by washing the paper strips for 15 min after electrophoresis with ethyl acetate. After localization of the different U.V.-absorbing fractions on the paper strips, they were cut out and eluted with d.s. illed water and concentrated to small volumes (200–500 μl) by lyophilization. The bases in the nucleotides were analyzed according to WYATT[10,11].

The spectrophotometric properties of the bases were determined after elution with a Unicam Spectrophotometer SP 500. The molar extinctions and the extinctions at different wavelengths as given by several authors[12] were used.

RESULTS AND DISCUSSION

In a number of experiments the quantitative reproducibility of the method was studied. Known amounts of AMP*, ADP and ATP were separated (Fig. 1) and after electrophoresis the nucleotides were localized, eluted from the paper and the extinction of the solution measured at 260 mμ. In Table I the results of these experiments are summarized. The data show that a quantitative recovery is obtained. Decomposition of ATP and ADP during the electrophoresis can be excluded.

The separation of nucleotides from acid-soluble extracts from thymus is shown in Fig. 2a and b. The nucleotides were identified by comparison of the relative mobilities of the fractions with those of reference compounds and by paper chromatographic analysis of the purines and pyrimidines obtained by hydrolysis of the nucleotides with formic acid. The results are summarized in Table II.

In a number of experiments the purity of the ADP- and ATP-fractions was further investigated. These fractions (9 and 11, Fig. 2) were eluted and analyzed. The results of the experiments are summarized in Table III.

---

* Abbreviations used: A, adenine; G, guanine; C, cytosine; T, thymine; U, uracil; 5-Me-C, 5-methylcytosine; H, hypoxanthine; AMP, ADP and ATP, the mono-, di- and triphosphates of adenosine; GMP, GDP and GTP, the mono-, di- and triphosphates of guanosine; CMP, CDP and CTP, the mono-, di- and triphosphates of cytosine; UMP, UDP and UTP, the mono-, di- and triphosphates of uridine; 5-Me-CMP and 5-Me-CTP, the mono- and triphosphates of 5-methylcytosine; UDPG, uridine diphosphate glucose; UDPGA, uridine diphospho-N-acetyl-glucosamine; TTP, thymidine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; E_{260}, extinction at 260 mμ wavelength. The reference compounds used in these experiments were obtained from Nutritional Biochemical Corporation, California Corporation for Biochemical Research and Sigma Chemical Company.

TABLE I

QUANTITATIVE SEPARATION OF MIXTURES OF AMP, ADP AND ATP BY ELECTROPHORESIS*

(see Fig. 1)

AMP	*ADP*	*ATP*
0.228	0.225	0.378
0.223	0.230	0.398
0.245	0.228	0.392
0.230	0.238	0.385

* To the electrophoresis strips were consecutively applied: 10 μl of an AMP solution, 20 μl of an ADP solution and 30 μl of an ATP solution. The solutions contained 2 mg of material per ml. Electrophoresis was carried out for 1 h at 2000 V, pH 3.6. The figures represent extinctions at 260 mμ of solutions of AMP, ADP and ATP after elution in 5 ml 0.01 *M* phosphate buffer pH 7.0. The extinctions of 10 μl of the AMP solution, 20 μl of the ADP solution and 30 μl of the ATP solution, after dilution to 5 ml with 0.01 *M* phosphate buffer, pH 7.0 were, 0.230, 0.239 and 0.386, measured at 260 mμ.

TABLE II

BASE COMPOSITION AND MOBILITIES OF THE NUCLEOTIDE FRACTIONS OBTAINED AFTER ELECTROPHORESIS OF AN ACID-SOLUBLE EXTRACT FROM THYMUS

(see Fig. 2a and b)

Fraction number	*Bases detected*	*Mobility fraction*	*Mobility ref. nucleotides**
1 and 2	H, U, A	1.8; 3.8	mixtures of ribosides and bases; mobilities very low.
3	C, 5-Me-C	13.3	13.6: CMP
4	A, 5-Me-C, C	19.2	18.0: DPN
5	A	23.7	23.7: AMP
6	—	30.3	—
7	G, A, C	41.6	40.0: GMP; 43.7: TPN; 50.8: CDP
8	H	46.1	50.7: IMP
9	A, U	53.4	52.5: ADP; 56.0: UMP
10	G	63.8	64.3: GDP
11	A, C, 5-Me-C, U	72.4	72.4: ATP; 74.5: CTP; 72.0: UDPG; 69.8: UDPGA
12	G, U	79.2	82.3: UDP; 79.5: GTP
13	U, T	97.9	100.0: UTP; 89.1: ITP; 99.5: TTP

* The figures were obtained by multiplying the original mobilities of the reference nucleotides (measured as a migration from the start) with a factor to render the mobility of UTP 100. In separations from acid-soluble extracts, the UTP band is usually rather broad. In these cases it is therefore more accurate to base the relative mobilities of the nucleotide fractions on the relative mobility of ATP (column 3).

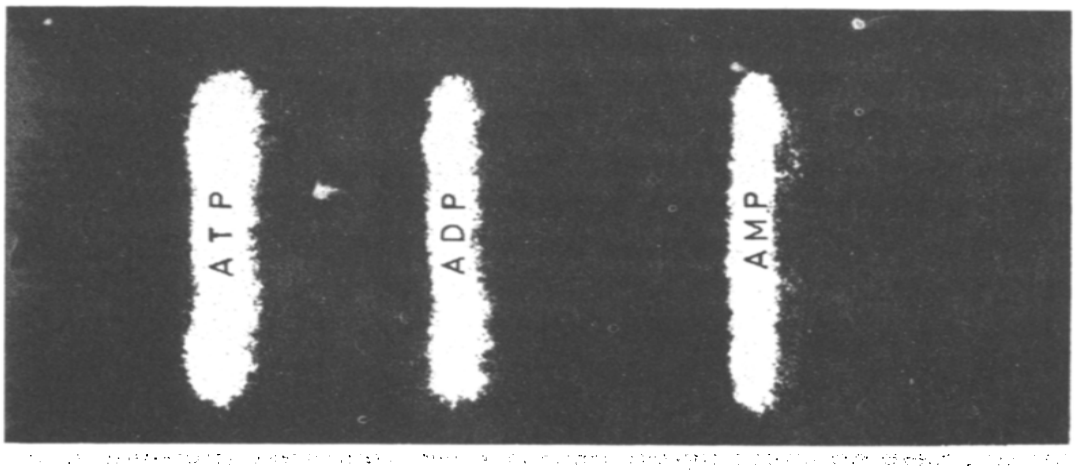

Fig. 1. Separation of AMP, ADP and ATP. Electrophoresis 1 h, 2000 V, pH 3.6.

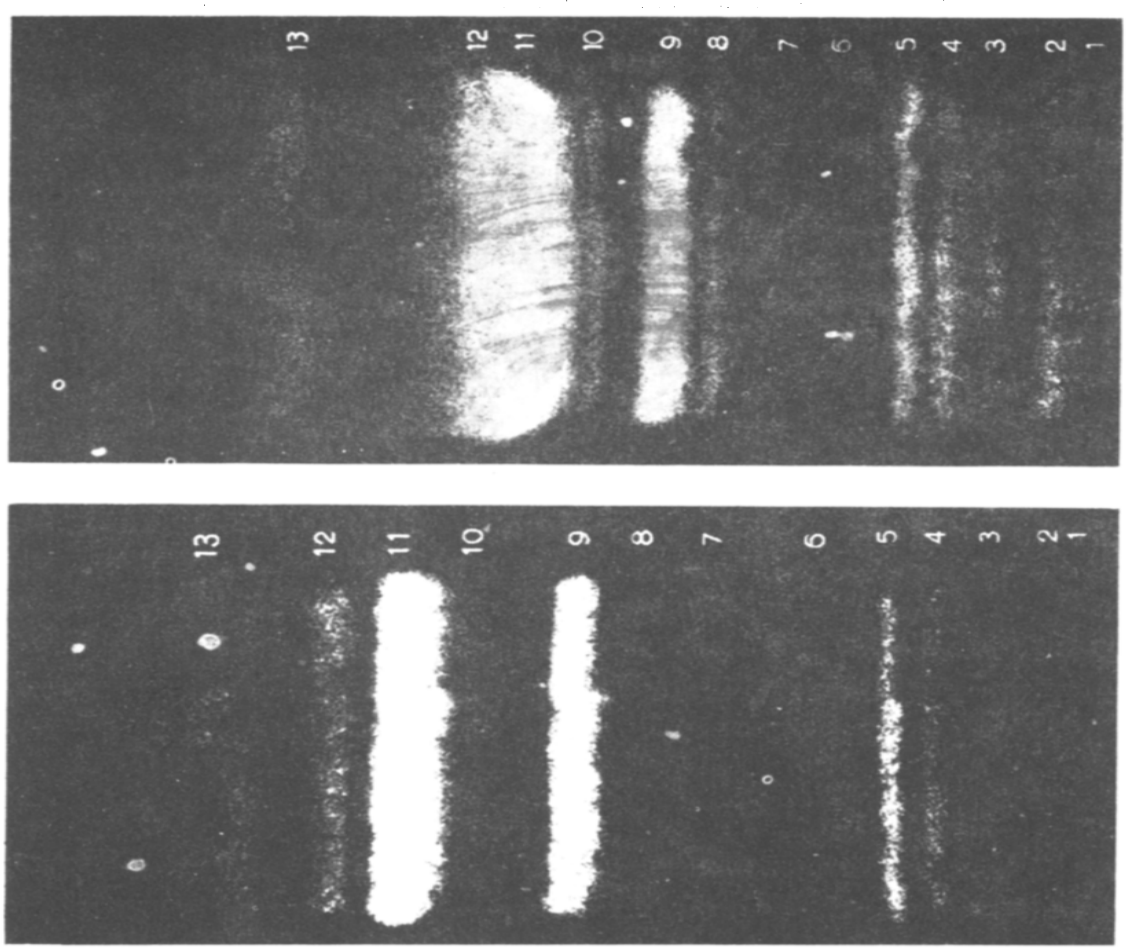

Fig. 2. Separations of acid-soluble extracts from thymus. Electrophoresis 1 h (a) and 3/4 h (b), 2000 V, pH 3.6. 1 and 2: ribosides and free bases. 3: CMP, 5-Me-CMP. 4: DPN, CMP, 5-Me-CMP. 5: AMP. 6: Not identified. 7: TPN, GMP, CDP. 8: IMP. 9: ADP, UMP. 10: GDP. 11: ATP, CTP, 5-Me-CP, UDPG, UDPGA. 12: GTP, UDP. 13: UTP, TTP.

TABLE III

PURITY OF ATP- AND ADP-FRACTIONS FROM ACID-SOLUBLE EXTRACTS FROM THYMUS

	Analyses of bases	
	Bases detected	*Purity** %
ATP-fractions; charcoal column treatment	No. 1: A, C, 5-Me-C, U	87
	No. 2: A, C, 5-Me-C, U	86
	No. 3: A, C, U, G	90
ADP-fractions; charcoal column treatment	No. 1: A, U	90
	No. 2: A, U	89
	No. 3: A, U	91
ATP-fractions; no charcoal column treatment	No. 1: A, C, 5-Me-C, U	72
	No. 2: A, C, 5-Me-C, U, G	62
ADP-fractions; no charcoal column treatment.	No. 1: A, U	79
	No. 2: A, U	78

* The purity of the ATP- or ADP-fractions is expressed as:

$$\frac{E_{\text{adenine } 260}}{E_{\text{bases detected } 260}} \cdot 100\,\%.$$

Among the bases 5-Me-C was found in a number of analyses. The amount, expressed in the same way as the purity of ATP, varied from 2–3 %. In some experiments 5-Me-C could not be detected.

It can be seen that the purities increase when the acid-soluble extracts are purified by charcoal column treatments before electrophoresis.

The results show that the general features of these acid-soluble extracts are very similar. All these "nucleotide-spectra" are characterized by relatively high amounts of ATP and ADP (see also Fig. 3). The purity of these fractions, especially that of the ATP- and ADP-fractions, was investigated in the case of the acid-soluble extract from thymus. The ATP- and ADP-fractions contain considerable amounts of other nucleotides; the ATP-fractions contain CTP, 5-Me-CTP, glucose-derivatives of UDP and in some tissues GDP; the ADP-fractions contain only UMP. The separation of some nucleotides by electrophoresis is almost impossible because of the small differences in relative mobilities (see Table II; compare the relative mobilities of ATP, CTP, UDPG and UDPGA, of GMP and TPN, of UTP and TTP). UDP and GTP were separable in several cases, in other cases the separation was not distinct. The occurrence of TTP in the acid-soluble extract, first mentioned by POTTER *et al.*[13], was confirmed in these experiments. In this case we could detect deoxyribose, the other fractions contained ribose.

One "nucleotide" (fraction No. 6), appearing in very small amounts directly

above AMP, was not identified. No purine or pyrimidine could be detected after formic acid treatment of this U.V.-absorbing, phosphate-containing compound.

The removal of pyridine from the electrophoresis strips was sometimes not complete. It was helpful to introduce an ethyl acetate wash of the paper strips in later experiments. The advantage of charcoal column treatment as regards the homogeneity of

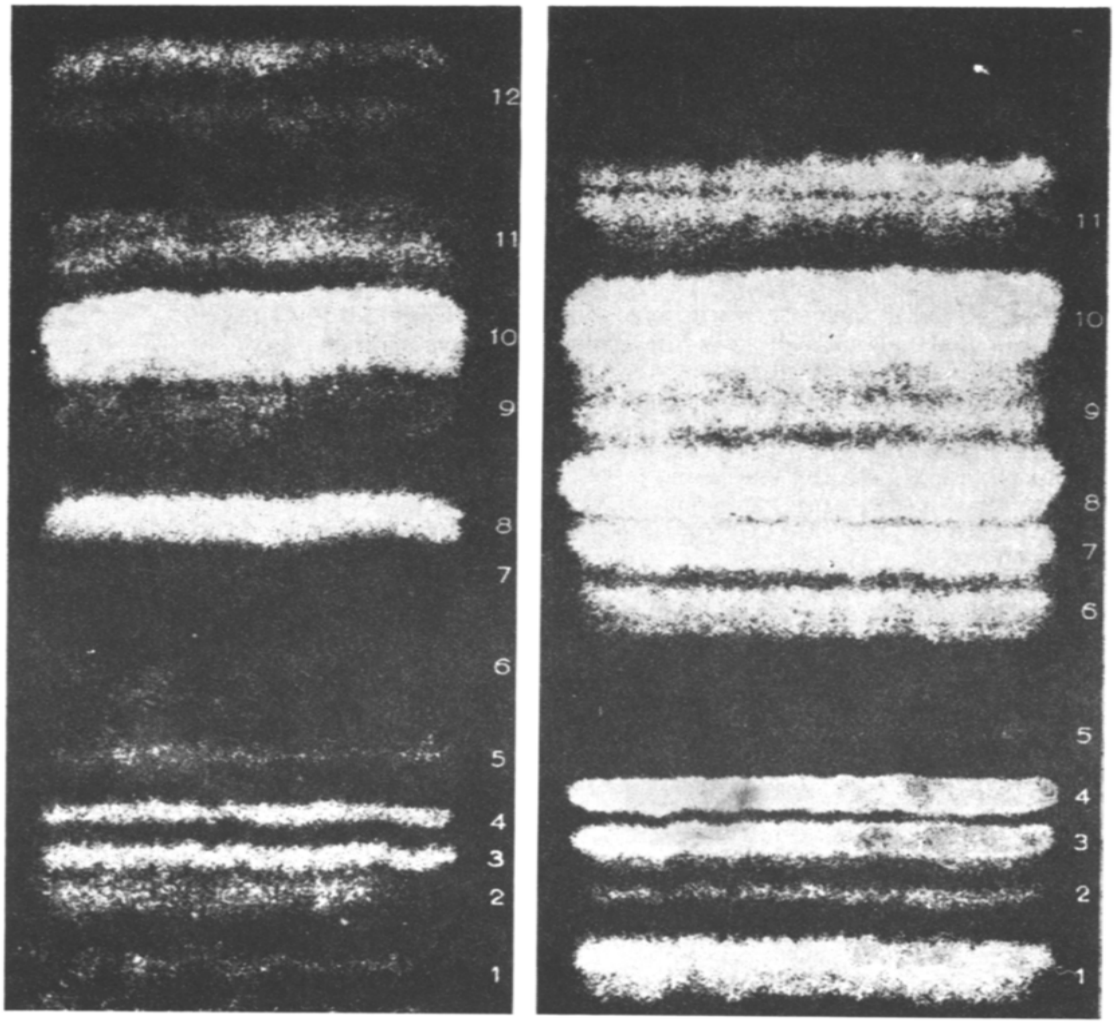

Fig. 3. Separations of acid-soluble extracts. Electrophoresis 1 h, 2000 V, pH 3.6. (a) ascites tumor. (b) liver. 1: ribosides and free bases. 2: CMP. 3: DPN. 4: AMP. 5: Not identified. 6: GMP, TPN. 7: IMP. 8: ADP. 9: GDP. 10: ATP. 11: GTP, UDP. 12: UTP.

the fractions obtained after electrophoresis, is obvious (see Table III). Other investigators have reported similar results[14]. In general the separations are better after a charcoal column treatment. It is likely that this procedure removes most of the contaminating non-nucleotide materials (*e.g.* salts, organic phosphate compounds) from the nucleotide mixture, which interfere with the separation of nucleotides.

The method presented has the advantage that nucleotides from acid-soluble tissue extracts can be quickly separated in many fractions under conditions which minimize the decomposition of acid labile di- and triphosphates.

SUMMARY

A method is presented for the analysis of nucleotides present in acid-soluble extracts from animal tissues. The method is based on the principle of high-voltage electrophoresis and consists in application of this method to acid-soluble extracts isolated from animal tissues.

The nucleotide pool is separated into 12–13 fractions; the contents and purity of these fractions are further investigated.

REFERENCES

1 R. B. HURLBERT, H. SCHMITZ, A. F. BRUMM AND V. R. POTTER, *J. Biol. Chem.*, 209 (1954) 23.
2 H. G. PONTIS AND W. L. BLUMSON, *Biochim. Biophys. Acta*, 27 (1958) 618.
3 B. MAGASANIK, *J. Biol. Chem.*, 186 (1950) 37.
4 J. N. DAVIDSON AND R. M. S. SMELLIE, *Biochem. J.*, 52 (1952) 594.
5 R. BERGKVIST, *Acta Chem. Scand.*, 11 (1957) 1465.
6 F. TURBA, H. PELZER AND H. SCHUSTER, *Z. physiol. Chem.*, 296 (1954) 97.
7 D. W. VAN BEKKUM, *Biochim. Biophys. Acta*, 25 (1957) 487.
8 A. P. RYLE, F. SANGER, L. P. SMITH AND R. KITAI, *Biochem. J.*, 60 (1955) 541.
9 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294.
10 G. R. WYATT AND S. S. COHEN, *Biochem. J.*, 55 (1953) 774.
11 G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
12 G. R. WYATT, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. 1, Academic Press Inc., New York, 1955, p. 262; G. H. BEAVEN, E. R. HOLIDAY AND E. A. JOHNSON, in E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. 1, Academic Press Inc., New York, 1955, p. 493.
13 R. L. POTTER, S. SCHLESINGER, V. BUETTNER-JANUSCH AND L. THOMPSON, *J. Biol. Chem.*, 226 (1957) 381.
14 K. K. TSUBOI AND T. B. PRICE, *Arch. Biochem. Biophys.*, 81 (1959) 223.