SEPARATION AND QUANTITATIVE DETERMINATION OF ADENINE NUCLEOTIDES AND URIC ACID BY MULTIPLE AND BY CONTINUOUS ASCENDING PAPER CHROMATOGRAPHY

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Methods for the quantitative determination of microgram amounts of nucleotides and related compounds are not altogether satisfactory. During the last few years RANDERATH and his group have published a number of papers describing improvements in resolution of purines, nucleosides and nucleoside phosphates by a variety of methods using paper and thin-layer chromatography[1] (TLC). A fairly good resolution has been obtained on layers of ECTEOLA- or DEAE-cellulose[2,3] and a still better resolution on layers prepared from cellulose impregnated with polyamide[4,5]. Quite recently the last mentioned procedure was adapted by RANDERATH AND RANDERATH[6] for quantitative microdetermination of ATP and other nucleotide coenzymes. Although the results obtained by the authors with mixtures of pure substances appear to be very good, the method does not seem to be quite suitable for examination of free nucleotides and related compounds in biological material. The concentration of these substances in animal tissues is usually extremely low and it is therefore practically impossible to obtain tissue extracts which, in the few μl used for spotting in TLC, would contain a sufficient amount of the substances necessary for quantitative determination. On the other hand, paper chromatography allows application of much larger volumes of solutions and, hence, also larger amounts of the examined substances.

The aim of the present investigation was to modify ascending paper chromatography, chosen for its simplicity, in order to make it suitable for quantitative determination of microgram amounts of nucleoside mono- and polyphosphates, and related compounds, in animal tissues.

In a preliminary study we tried first of all the unidimensional multiple development ascending paper chromatographic technique (MAC) of JEANES *et al.*[7], which as early as 1951 had been worked out in detail to improve the resolution of sugars having similar R_F values. The theory and the advantages of MAC have been discussed recently by THOMA[8], LENK[9] AND RÜDIGER AND RÜDIGER[10].

Using MAC for the separation of microgram quantities of some adenine nucleotides with a solvent mixture prepared according to RANDERATH AND STRUCK[11], we obtained after three or four runs an excellent resolution of AMP, ADP and ATP. However, when uric acid was also present the method failed completely. This was due to the fact that the behaviour of uric acid differed strikingly from that of the nucleotides; namely, during the second and the subsequent runs most of the uric

acid did not move any further and, as a consequence, the nucleotides gradually overlapped the spots of uric acid and the whole procedure could lead to false results.

In search of a better method of separation of all the substances under investigation we chose the technique of continuous ascending paper chromatography (CAC), first proposed and highly recommended by FISCHBACH AND LEVINE[12]. After a number of preliminary experiments the following procedure with two successive solvent mixtures appeared to give a good resolution of ATP, ADP, AMP and uric acid and to allow a quantitative spectrophotometric determination of these substances in amounts from 0.02 up to about 0.10 μmole of each of them. For the first run *n*-propanol–1 % NH_3 (60:40, v/v)[13] was used; this also permitted the detection of the faster moving purines and nucleosides. After drying the paper at room temperature, the second run was performed in a solvent mixture prepared according to RANDERATH[11] and a good resolution of the nucleotides and uric acid was obtained. The details of the method are described below.

EXPERIMENTAL

Whatman No. 1 paper was used throughout the experiments. The paper was washed continuously for 10–15 h with 0.4% EDTA solution in a glass jar with a slit in the cover using a similar procedure to that used for continuous ascending chromatography (see below): about 10 (or even more) sheets of paper were pressed together and washed in a single run. After washing the sheets were dried in air and, afterwards, washed twice in a similar manner with distilled water; they were again dried in air and preserved between two glass plates. As a rule the outer sheets were discarded or only used for preliminary experiments. The method described for washing the paper appeared to be simpler and more handy than other conventional methods.

The size of chromatograms in the multiple ascending chromatography (MAC) and in continuous chromatography (CAC) was different: in the first case it was 18 ×

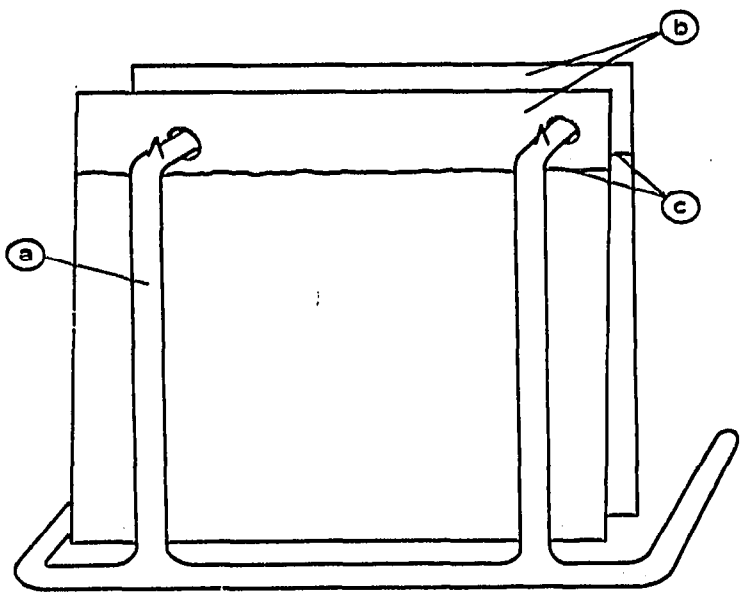

Fig. 1. Arrangement for multiple ascending paper chromatography. a = The glass support; b = the chromatograms; c = the level to which the solvent moves during each of the runs.

18 cm and in the second 18 × 22 cm, respectively. Two holes were made in the upper part of the paper by a perforator in order to hang the paper on a glass support during MAC (Fig. 1) or to fix it additionally during CAC (Fig. 2).

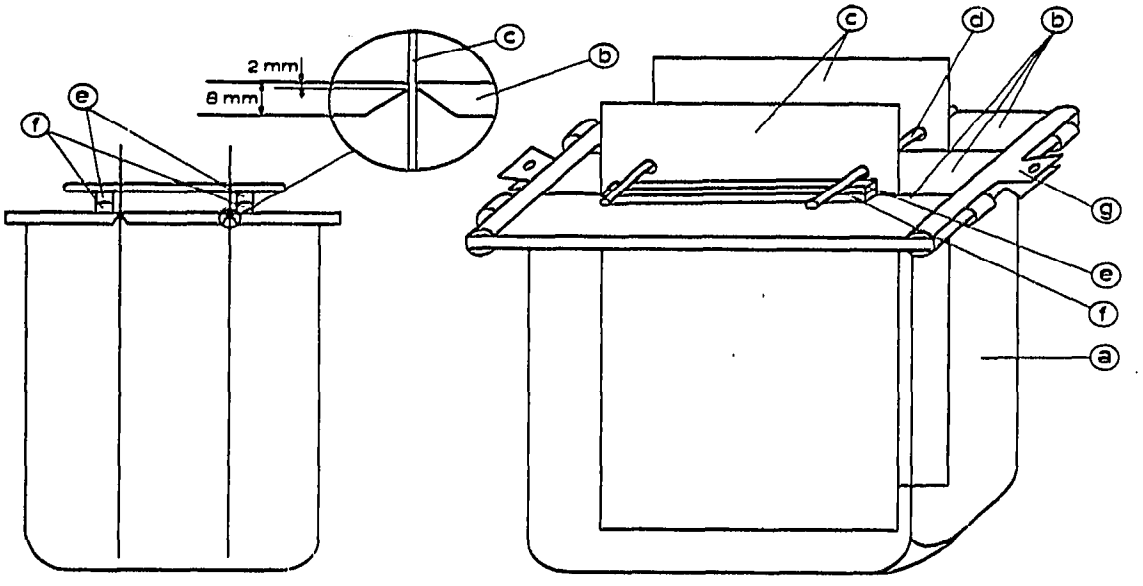

Fig. 2. Arrangement for continuous ascending paper chromatography. On the left, the side view of the jar; in the middle, a magnified view of the place where the chromatogram is fixed between the gaflon plates; on the right, a general view of the whole arrangement. a = The glass jar; b = gaflon plates used as cover; c = chromatograms; d = glass rods; e and f = narrow glass plates used for supporting the rods; g = fasteners.

The chromatographic development was performed in a rectangular glass jar (20 × 12 × 20 cm high) with polished surface covered with a well fitting ground glass cover (20 × 12 cm), for MAC; or with three tightly adjoining teflon or gaflon plates 24 × 4.5 × 0.8 cm thick, for CAC. Because minute droplets of the solvent sometimes condensed on the lower side of the cover, which in the case of CAC could cause the undesirable effect of a sudden local rise of the solvent concentration on the paper at any place touching the slit, it was very useful to grind the edges of the gaflon plates in a special manner: the whole arrangement used for CAC is shown in Fig. 2. When the solvent mixture reaches the edge of the cover it evaporates in the air, and as a result, a further amount of the solvent is forced to ascend. Towards the end of the procedure, a fairly straight or a wavy line becomes visible at the boundary; it most probably consists of small amounts of impurities which were still present in the paper and the solvents.

The arrangement shown in Fig. 2 makes allowance for the equilibration of the paper in the atmosphere of the solvent vapour, if necessary. The sheets of paper may be hung a few millimeters above the level of the solvent on two glass rods supported on two pairs of narrow glass plates (about 8 mm thick); when two glass plates, one from each side of the chromatograms (Fig. 2 e), are taken out the sheets of paper can be lowered and dipped into the solvent mixture.

Preliminary experiments showed that equilibration did not improve the resolution of the investigated substances and it was therefore omitted; it appeared useful,

however, not to support the glass rods directly on the gaflon covers but somewhat above them on two narrow glass plates shown in Fig. 2f.

The solutions of the samples to be analysed (in all cases the pH was adjusted to about 7) were placed as drops on the paper (at a distance 2.5 cm from the lower edge and 3 cm from each other) by gently expelling a few μl portions of the liquid from a micropipette; this was done in a gentle current of air to obtain the minimum size of the spot. The exactly measured total volume of the spotted solutions could be varied, depending on the quantity of the substances to be analyzed, from 5 to 200 μl by using pipettes of the appropriate volumes. A very simple and easily made wooden frame was used for spotting, the paper being firmly held during spotting between 4 glass plates of appropriate size.

ATP, ADP and AMP were purchased from Boehringer; adenine, xanthine and adenine nucleoside were obtained from Fluka, and uric acid from B.D.H.

In the CAC procedure, as mentioned above, two solvent systems were used successively. The first (solvent A) was *n*-butanol–1 % NH_3 (60:40)[13], and the second (solvent B) was *n*-butanol–acetone–5 % NH_3–acetic acid–water (90:30:20:20:40; v/v)[11]. In MAC solvent B was used exclusively. Acetone, *n*-butanol and ammonia were chemical grade Polish products; the organic solvents as well as H_2O were redistilled before use.

After development the substances under investigation were visualized under a short wave U.V. lamp. The detection was made significantly easier if the sheet of paper, after drying in air, was dipped for 1–2 sec into about 0.0003 % solution of Rhodamine B in absolute alcohol. After this procedure (and after evaporation of the alcohol) the dark spots became much more distinct on the fluorescent background of the paper. As has been shown in a special series of experiments the presence of the small amounts of Rhodamine B remaining in the paper had practically no effect upon the spectrophotometric quantitative determination performed on the substances after their elution.

The detected spots were outlined under U.V. with a soft pencil and, afterwards, were cut out as rectangles 3 cm wide and 1 to 3 cm long. Next, each of them was cut into 3 to 5 pieces which were placed together in a small crystallizer.

The following procedure for quantitative elution of the investigated substances has been found to be simple, quick and highly efficient. By means of a calibrated 2–3 ml dropper (with a small rubber bulb) about 0.5 ml of hot water, containing a minute quantity of Li_2CO_3 (about 0.002 %, pH about 7) was poured over the pieces of paper in the crystallizer. The liquid was sucked back and poured again several times, and was finally filtered through a small glass filter (Schott G-1) into a narrow, calibrated (3 ml) glass tube with a widened neck. The procedure was repeated 3–4 times with fresh 0.5 ml portions of hot water and, at the end, the whole eluate was diluted to the mark. The blanks were prepared in a similar way by elution of a similar strip of the chromatogram which did not contain the analyzed substances.

In the CAC procedure each of the two developments (with solvents A and B, respectively) took 17 h. This period of time (from 4 p.m. till 9 a.m. of the next day), chosen from a purely practical point of view, gave very satisfactory results at the same time. When using the MAC procedure each of the four runs lasted about 2 h; this allowed migration of the solvent to the level shown in Fig. 1c.

RESULTS AND DISCUSSION

The efficiency of the elution procedure was checked for ATP, ADP, AMP and uric acid. Exact volumes of the solutions (usually 20 μl), containing various amounts of the substances under investigation, were placed upon Whatman No. 1 paper with a micropipette. After drying in air 3 × 3 cm squares around each of the spots, and similar squares from unspotted paper (for blanks), were cut out and eluted as described in the previous section. A Beckman spectrophotometer was used to determine the extinction value at 259 mμ for the nucleotides and 290 mμ for uric acid, respectively. The results were compared with those obtained with samples of the same solutions measured directly into the volumetric glass tubes; in the latter case the blanks contained pure water. The specific absorption for the nucleotides at pH 7 was accepted as 15000[14] and for uric acid 12200[15], respectively.

It was found that from 0.02 up to 0.2 μmole practically the whole amount of each nucleotide, as well as the uric acid, was recovered.

Unidimensional multiple ascending chromatography (MAC)

The results obtained by this technique with ATP, ADP and AMP are presented in Fig. 3a. It can be seen that after 3 runs the nucleotides were distinctly separated and the spots obtained became flattened; the latter fact has already been noticed earlier[9].

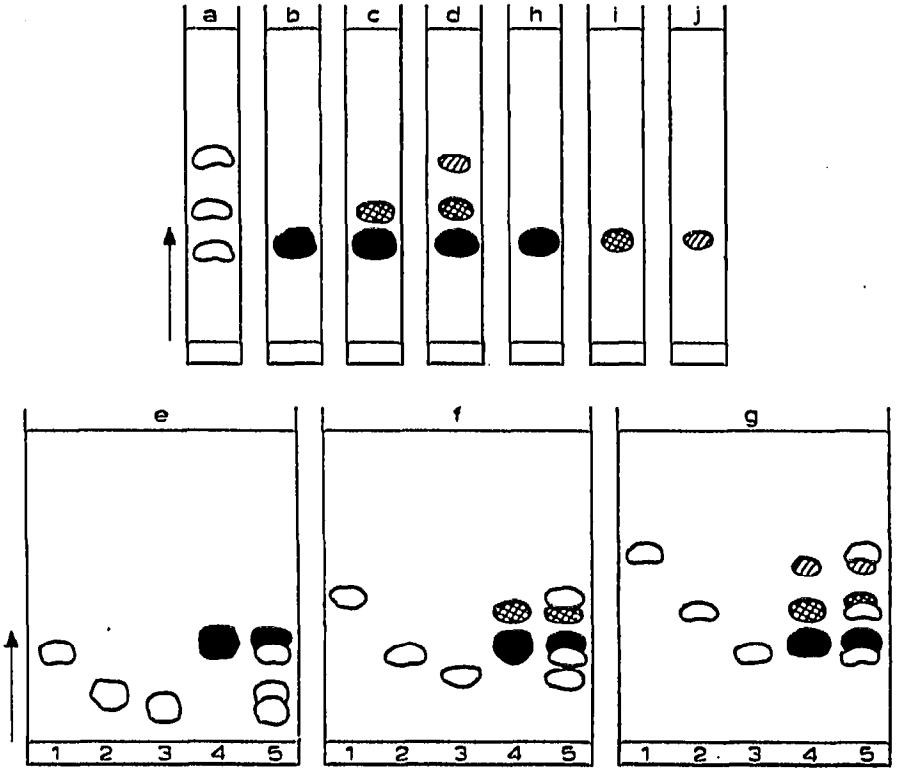

Fig. 3. Multiple ascending chromatography. a = AMP, ADP and ATP after three runs; b, c and d = uric acid after one, two and three runs, respectively; e, f and g = AMP (1), ADP (2), ATP (3), uric acid (4) and a mixture of the nucleotides and uric acid (5) after one, two and three runs, respectively (the positions of uric acid are shown as black or dashed spots); h, i and j = rechromatography of the substances eluted from the 3 spots shown in d. About 0.05 μmole of each of the substances was used in this series of analyses.

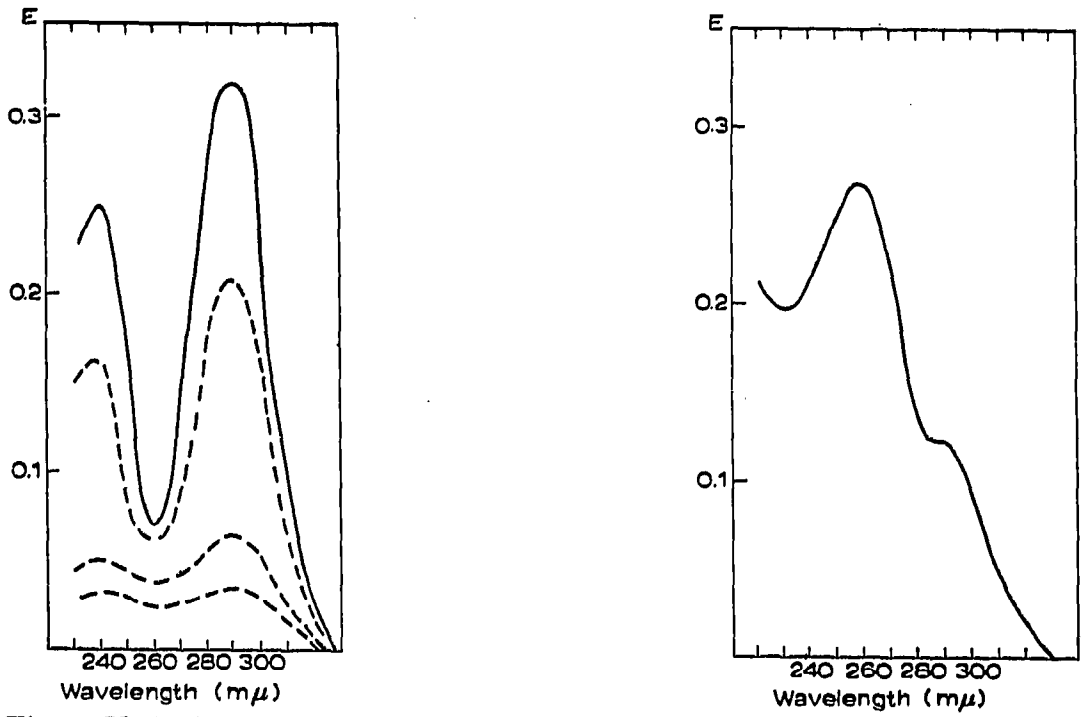

Fig. 4. U.V. absorbance of uric acid measured directly (upper continuous curve), and absorbance of the substances eluted from the three spots (dashed curves) shown in Fig. 3d. The amount of uric acid used for the chromatographic separation was identical to that used for direct determination.

Fig. 5. U.V. absorbance of the substances eluted from one of the three spots shown in Fig. 3g (5).

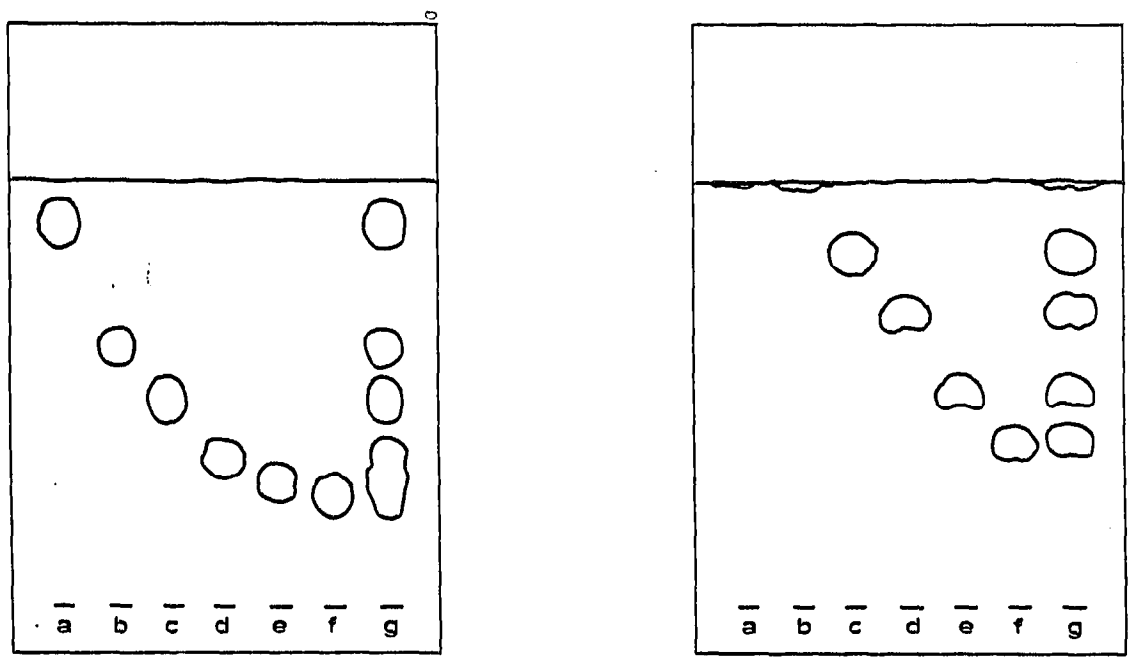

Fig. 6. Continuous ascending chromatography. Results obtained after separation in the solvent A. a = Adenine + adenosine; b = xanthine; c = uric acid; d = AMP; e = ADP; f = ATP; g = a mixture of all the substances mentioned.

Fig. 7. Continuous ascending chromatography. Results obtained after the subsequent separation in solvent B. The letters a–g indicate the same substances as shown in Fig. 6. About 0.05 μmole of each of the substances was used.

In contrast, uric acid behaved quite differently. Figs. 3b–g summarize the results obtained with solutions containing either uric acid alone (Figs. 3b–d) or uric acid and a mixture of nucleotides (Figs. 3e–g). Fig. 4 shows the U.V. absorption spectrum of a definite amount of directly measured uric acid (upper curve) and the absorption spectra (dashed curves) of the substances from the three spots shown in Fig. 3d obtained from the same amount of uric acid after three runs.

To ensure that the new faint spots of uric acid, appearing after the second and the third run (Figs. 3c and d), contain the same compound and not any different tautomeric form, or perhaps even another substance, the three spots shown in Fig. 3d were eluted separately and rechromatographed in the solvent B. Figs. 3h–j indicate that in all three cases it was most probably the same substance, namely uric acid; hence it may be concluded that when the MAC technique was used only a small part of the uric acid moved any further during subsequent developments.

From the above data it is evident that when uric acid is present in a solution containing nucleotides the determination of all these substances by MAC may provide completely wrong results (see Figs. 3e–g). Fig. 5 shows the U.V. absorption of one of the spots shown in Fig. 3g (5). It is clear that the spots of the nucleotides become contaminated with uric acid. Without going into details concerning the reasons for the observed differences in behaviour of uric acid when compared to that of the nucleotides, which are not quite clear, it must be concluded that, in this case, MAC is acceptable only when the behaviour of each of the particular substances under investigation has been previously studied. It seems therefore that although the technique itself may sometimes have great advantages it is not sufficient to base it only upon the R_F values, which is what is generally proposed[8–10].

Continuous ascending chromatography (CAC)

The results of a separation of some nucleotides and related substances by CAC with the solvent A (in a manner described in the experimental section) are presented in Fig. 6. It can be seen that adenine and adenosine, which hardly separate under these conditions, have the highest "relative" R_F values. (It is obvious that the ordinary R_F cannot be determined in a continuous procedure.) The rate of movement of xanthine and of uric acid is much lower and these substances are distinctly separated from adenine and from each other; they have also been identified by their absorption spectra. AMP moves somewhat more slowly than the purines, and in solvent A is hardly separated from ADP and ATP; all these three substances can be seen under U.V. as one elongated spot.

A very distinct separation of uric acid, AMP, ADP and ATP was obtained after the subsequent continuous development performed in solvent B (Fig. 7). The absorption spectra of the substances eluted from the four spots shown in Fig. 7g are shown in Fig. 8.

A large number of quantitative determinations of AMP, ADP, ATP and uric acid were performed in a special series of experiments. From 0.02 to 0.1 μmole of each of the compounds was placed on the starting line on the same spot and the development was performed in solvents A and B, successively.

The results are summarized in Fig. 9. In most cases over 90 % of each of the nucleotides was recovered; the standard deviations being about ± 5 %. The recovery of uric acid was, however, somewhat lower and the standard deviations were, in some

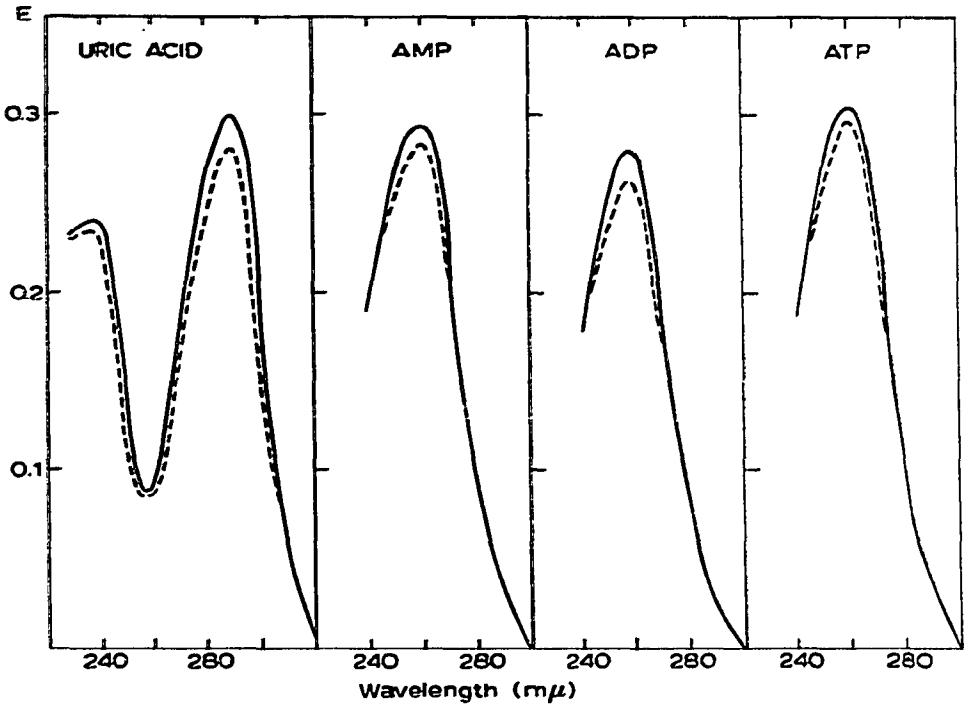

Fig. 8. U.V. absorbance of uric acid and nucleotides measured directly (upper continuous curves) or eluted from the four spots shown in Fig. 7g (dashed curves). Identical amounts of the corresponding substances were used for direct determination and for the CAC separation, respectively.

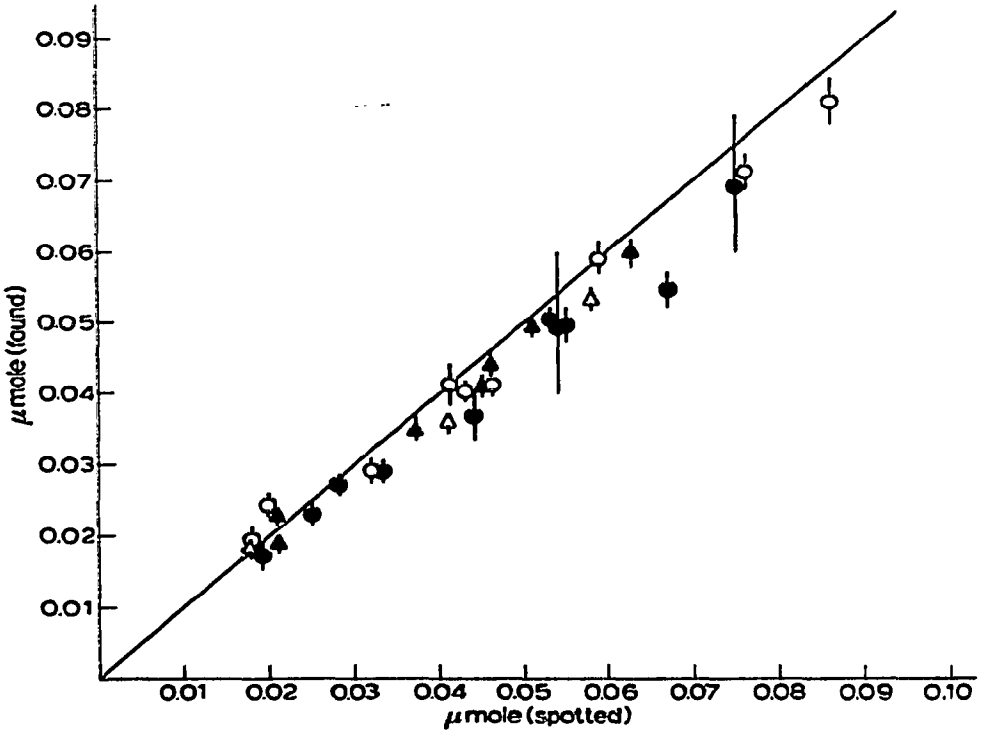

Fig. 9. Quantitative determination of various amounts of nucleotides and uric acid after separation by continuous ascending chromatography in solvents A and B successively; mean values and S.D. from 4–6 analyses. O = AMP; △ = ADP; ▲ = ATP; ● = uric acid.

cases, distinctly higher. Moreover, tentative experiments (not shown in the figure) indicated that if the quantity of the uric acid spotted exceeded 0.1 μmole its recovery after chromatographic separation became still less efficient.

In order to determine whether the substances analyzed undergo degradation during the whole procedure used in the present method a special series of experiments was performed. To a mixture of uric acid, AMP, ADP and ATP a small amount of [^{14}C]ATP was added and the whole procedure of chromatographic separation of these substances was performed in solvents A and B, successively. The strips of chromatograms were counted (from the starting point up to the front) with the use of a Radiochromatogram Scanning System attached to the Nuclear Chicago Corporation Gas Flow Counter with "Micromil" window.

Fig. 10 shows that practically the whole of the radioactivity was found exclusively in the spot of ATP. This indicates that even ATP, which is probably the most labile of the substances present in the mixture, did not break down at all.

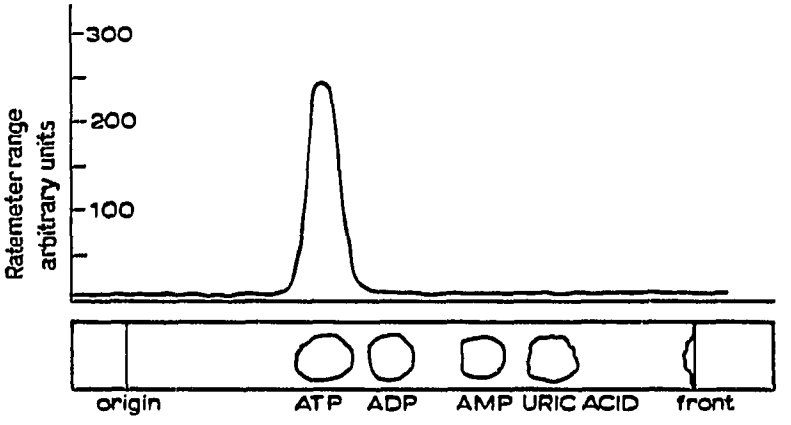

Fig. 10. Radioactivity found in the strip of paper after separation of AMP, ADP, ATP (containing [^{14}C]ATP) and uric acid by continuous ascending chromatography with solvents A and B, successively.

It seems that the CAC procedure described in the present paper may be helpful in examination of biological material; it has already been applied in our investigations of nucleotide and uric acid metabolism in insects (to be published). Accumulation of large amounts of uric acid in the insect body during some developmental stages made it impossible to use the most recent modification for resolution of complex nucleoside triphosphate mixtures described by NEUHARD *et al.*[16]. The last mentioned method requires lyophilization of the extract, and this procedure, we have found, results in the loss of the greater part of the uric acid, most probably because of its very low solubility. Also, the possibility cannot be excluded that during lyophilization a partial degradation of some of the labile compounds may take place.

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SUMMARY

It has been found that when multiple ascending chromatography is used, the movement of particular substances must be checked beforehand because some of them, *e.g.* uric acid can cause an overlapping of the spots and misleading results.

A simple procedure of continuous ascending paper chromatography is described which permits a good separation and quantitative determination of microgram amounts of AMP, ADP, ATP and uric acid present in the same sample.

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