*Journal of Chromatography*, 79 (1973) 360–363


CHROM. 6637

**Note**

## Determination of acid-soluble nucleosides and bases in myocardium by thin-layer methods*

In studying the free energy metabolism of myocardium during ischemia, we have developed thin-layer chromatographic techniques for determination of the major compounds involved. The technique for separation of the acid-soluble nucleotides, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and inosine monophosphate (IMP), by anion-exchange on polyethyleneimine (PEI) cellulose thin layers has been previously described[1]. This paper describes a method for determination of the degradation products of these nucleotides using the anion-exchange properties of PEI cellulose, and an $(NH_4)_2SO_4$-based solvent described by MARKHAM AND SMITH[2], and COFFEY AND NEWBURGH[3]. These compounds include the nucleosides, inosine and adenosine, and the nucleobase, hypoxanthine.

*Experimental*

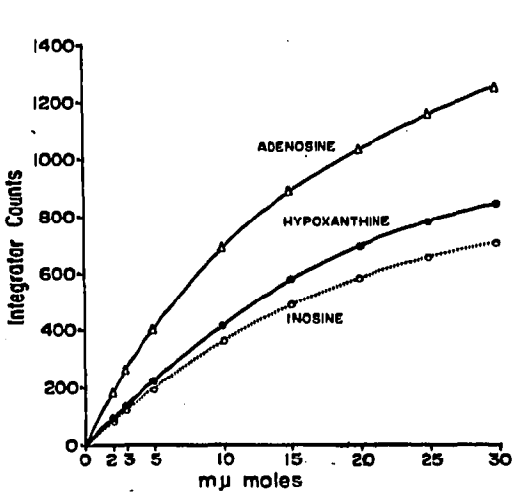
*Sample preparation.* The experimental protocol utilized an open-chest mongrel dog preparation, in which the heart was electrically fibrillated. Stainless-steel tongs, pre-cooled in liquid nitrogen were clamped on to a portion of heart muscle at various times after fibrillation, freezing it *in situ*. The tissue was immediately excised and immersed in liquid nitrogen. This sample was then pulverized to a fine powder using a pre-cooled, stainless-steel mortar and pestle. A portion of this powder, weighing 300–500 mg, was added to a pre-weighed homogenizer tube containing 2 cc of 0.6 *N* perchloric acid. This mixture was homogenized by a motor-driven Teflon pestle for 2 min. The supernatant was removed after centrifugation at 2,000 r.p.m. for 20 min, and neutralized with 1 *M* KOH, and Bromthymol Blue as an indicator. The $KClO_4$ precipitate formed at neutralization was removed by additional centrifugation. The extract was then frozen until time for application to the thin-layer plates.

*Chromatography.* Quantitative reference curves were obtained by spotting quantities of from 2–30 m$\mu$moles of adenosine, hypoxanthine, and inosine on polyethyleneimine (PEI) cellulose plates, followed by development in distilled water. The plates were 20 × 20 cm pre-coated PEI-cellulose plates with a layer thickness of 0.10 mm (Brinkmann Instruments, Inc.). The cellulose coating contained a fluorescent dye (maximum wavelength absorption of 254 m$\mu$) for resolution of the UV absorbing compounds by fluorescence quenching. The nucleosides and bases were quantitated directly on the plate by scanning with a dual-beam spectrodensitometer (Schoeffel Instruments, Model SD 3000) having an adjustable monochromometer (Model QPM 30), and an optical density computer (Model SDC 300). Peak areas were measured by the integrating strip recorder (Model SDR 303), and a disc digital

* This work was supported by NIH Grant No. HE 11780.

readout printer (Model 610). The dual-beam feature compensates for differences in layer thickness, as only the ratio of the optical density is recorded. Plates were scanned at a wavelength of 260 m$\mu$ with a full scale deflection on the recorder chart of 0.4 optical density units. The relationship of the number of m$\mu$moles spotted to the number of integrator counts for a given peak area is shown in Fig. 1. These areas were determined from the integrator readout minus any baseline error. Each point on the curve represents the mean of five determinations. The probable error of quantities from 2–5 m$\mu$moles was *ca.* 4–6%, while quantities above this amount had a probable error of *ca.* 2–3%.

For sample determination, two plates were prepared by scoring the PEI-cellulose layer into 1-cm strips. Both plates were spotted with 20 to 30-$\mu$l aliquots of each sample extract applied to alternate strips under a stream of warm (50°) air. The samples were applied with 50-$\mu$l No. 705 Hamilton microsyringes in a semi-automated sample applicator device developed for this procedure. One plate was developed in 1.25 *N* saline for nucleotide determination as previously described[1]. The second plate was used for nucleoside and base determinations. Samples were applied 1.5 cm from the bottom of the plate, then the air-dried plate was developed in distilled water to a distance of 4.5 cm. This development moved the water-motile nucleosides and bases from the starting-line while the nucleotides remained bound at the starting-line by polyethyleneimine groups. The plate was again air-dried and the cellulose layer was scraped from the glass below a level of *ca.* 2.5 cm. This removed the nucleotides bound at the starting-point, leaving only nucleosides and bases. The plate was subsequently developed to a solvent front height of *ca.* 14 cm in saturated $(NH_4)_2SO_4$–1 *M* sodium acetate–2-propanol (80:18:2).



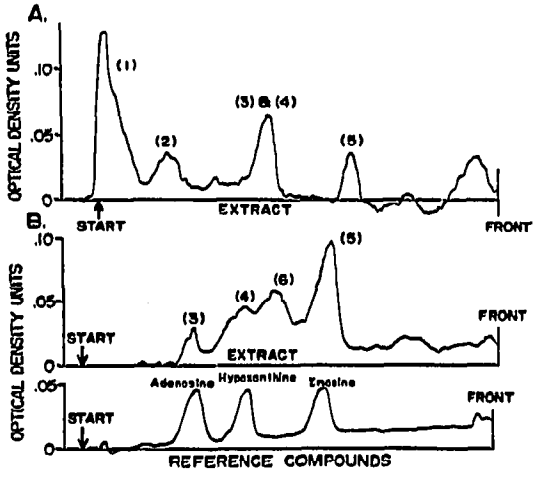




Fig. 1. Standard quantitative reference curves for adenosine, inosine and hypoxanthine at 260 m$\mu$. The relationship between the quantity spotted and the peak area in arbitrary integrator counts is shown.

Fig. 2. UV density scan of myocardial extracts on PEI-cellulose plates after development in: (A) distilled water and (B) distilled water followed by saturated $(NH_4)_2SO_4$–1 *M* sodium acetate–2-propanol (80:18:2). Peaks tentatively identified are: (1) nucleotides, (2) Phenol Red indicator, (3) adenosine, (4) hypoxanthine, (5) inosine, and (6) a UV-absorbing compound not yet characterized. Reference compounds for (B) are also shown.

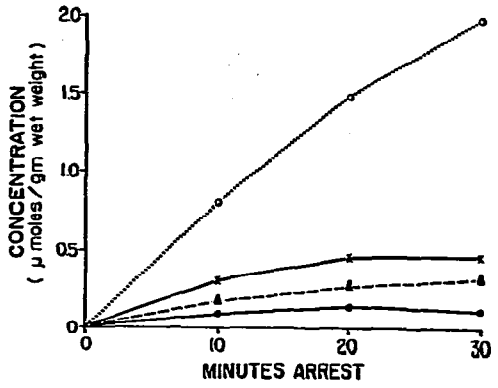



Fig. 3. Results of a typical experiment showing myocardial concentration of adenosine (●), hypoxanthine (×), and inosine (○), as well as the nucleotide IMP (▲) during a 30-min arrest period.

TABLE I

A COMPARISON OF TWO METHODS OF DETERMINATION OF ACID-SOLUBLE COMPOUNDS IN ANOXIC HEART MUSCLE

| *Investigator* | *Period of anoxia (min)* | *Inosine*[a] | *Hypoxanthine*[a] | *IMP*[a] | *Adenosine*[a] | *Animal* | *Methods* |
|---|---|---|---|---|---|---|---|
| IMAI *et al.* | 10 | 0.9 ± 0.2 | 0.2 ± 0.05 | 0.2 ± 0.05 | 0.2 ± 0.05 | rabbit | IMAI *et al.*[5]. Inosine, adenosine and hypoxanthine were determined by enzymatic methods. IMP was separated by column chromatography, quantitated by molar extinction at 248 mμ. |
| Present study | 10 | 0.88 ± 0.09 | 0.23 ± 0.05 | 0.23 ± 0.02 | 0.07 ± 0.03 | dog | |
| IMAI *et al.* | 20 | 1.65 ± 0.15 | 0.4 ± 0.05 | 0.2 ± 0.05 | 0.1 ± 0.05 | rabbit | |
| Present study | 20 | 1.50 ± 0.16 | 0.46 ± 0.1 | 0.32 ± 0.03 | 0.10 ± 0.02 | dog | |

[a] Expressed as micromoles per gram heart muscle.

The nucleosides and bases are motile in distilled water on PEI-cellulose layers[4], but the $R_F$ values of adenosine (0.43) and hypoxanthine (0.44), are sufficiently close that these compounds have been inseparable in aqueous solutions. Fig. 2 illustrates a UV density tracing of an extract of ischemic heart muscle developed on a PEI-cellulose plate demonstrating: (A) the inability of water development to separate adenosine and hypoxanthine, and (B) separation of these compounds with development in distilled water followed by satd. $(NH_4)_2SO_4$–1 *M* sodium acetate–2-propanol. Reference standards developed in the $(NH_4)_2SO_4$ based solvent are also indicated.

The absorption peaks were tentatively identified by $R_F$ values, which were: 0.24 for adenosine, 0.40 for hypoxanthine, and 0.59 for inosine. Additional confirmation was achieved by spectral absorption curves for each unknown compound. The peaks identified as adenosine had a maximum absorption wavelength of 260 m$\mu$, while those of hypoxanthine and inosine had absorption maxima of 250 m$\mu$.

A constant finding in the anoxia heart samples was a peak between hypoxanthine and inosine which had an absorption maximum of 260 m$\mu$. This peak has not been definitely identified, but may be an adenine-containing coenzyme.

*Results and discussion*

Fig. 3 shows the results of a typical experiment in which the myocardial concentrations of the adenine nucleotide degradation products were determined in triplicate during a 35-min period of cardiac arrest. IMP has been included with the nucleosides and bases, since it also accumulates during ischemia. The anion-exchange separation procedures for IMP and the adenine nucleotides have been previously reported[1]. None of these compounds is detectable in the normal heart, but they accumulate rapidly as the adenine nucleotides are broken down in anoxia. Of these compounds, inosine showed the greatest increase, reaching a value of approximately 2 $\mu$moles/g wet weight.

A comparison of the values obtained by this technique, and those obtained by IMAI *et al.*[5] using other methods, is presented in Table I. The results obtained by this method correlate well with those obtained by the method of IMAI *et al.* We feel this thin-layer technique for nucleoside and base determination, together with the method for nucleotide determination previously presented[1], offers a rapid and relatively simple technique for evaluation of the major compounds involved in the anaerobic metabolism of the high-energy adenine nucleotides.

*Department of Physiology and Biophysics,*
*University of Mississippi School of Medicine*
*Jackson, Miss. 39216 (U.S.A.)*

JAMES C. PARKER
CARL E. JONES
ELVIN E. SMITH

1 C. E. JONES, J. C. PARKER AND E. E. SMITH, *J. Chromatogr.*, 64 (1972) 378.
2 R. MARKHAM AND J. D. SMITH, *J. Biochem.*, 49 (1951) 401.
3 R. G. COFFEY AND R. W. NEWBURGH, *J. Chromatogr.*, 11 (1963) 376.
4 K. RANDERATH AND E. RANDERATH, *J. Chromatogr.*, 16 (1964) 111.
5 S. IMAI, A. L. RILEY AND R. M. BERNE, *Circ. Res.*, 15 (1964) 443.

First received November 21st, 1972; revised manuscript received February 7th, 1973