CHROM. 5770

Determination of myocardial acid-soluble adenine nucleotides on anion-exchange thin layers

We have studied myocardial energy metabolism during cardiac arrest and recovery. As part of these studies we have determined the concentrations of acid-soluble adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) in canine hearts by application of anion-exchange thin-layer chromatography. The basic chromatographic principles used in our study were first described by RANDERATH AND RANDERATH[1], and the methods by which these principles were applied in our research are described here.

Myocardial biopsy and preparation of myocardial extract

Cardiac arrest was produced in open-chest dogs by electrically induced fibrillation. Before arrest and at various times during arrest, small portions of left ventricle (100–200 mg) were rapidly frozen *in situ* with stainless-steel tongs, excised and immersed immediately in liquid nitrogen. The frozen muscle was pulverized with a precooled stainless-steel mortar and pestle, placed in a pre-weighed homogenizer tube containing approximately two volumes of 0.6 *N* perchloric acid (PCA) and homogenized with an electrically driven pestle for 3 min. Following homogenization the supernatant was separated by centrifugation at 2,000 *g* for 15 min. The supernatant was then neutralized with potassium hydroxide, and the precipitate was removed by recentrifugation. The supernatant was then used for determination of adenine nucleotides as described below.

Chromatography

For anion-exchange chromatography polyethyleneimine (PEI) impregnated cellulose precoated glass plates were used (Brinkmann Instruments, Inc.). Since the adenine nucleotides were to be quantitated directly on the plates by UV absorption, a short-wave UV indicator (maximum absorption at 254 mμ) was incorporated into the adsorbent layer. The plates were the standard 20 × 20 cm and had a thickness of 0.10 mm. Each plate was scored into strips of 1 cm in width for use in a dual beam spectrodensitometer.

To obtain reference curves, 5–10 μl of reference solutions for ATP, ADP, and AMP were spotted on alternate strips of scored plates. Samples were applied under a stream of warm air using a Hamilton 10-μl syringe. The reference solutions were of varying concentrations, and between 5 and 50 nmoles of each reference nucleotide was spotted. The spotted chromatogram was then developed in 1.25 *M* NaCl for a distance of *ca.* 12 cm. All chromatography was performed in closed glass tanks. The developed chromatogram was air dried and viewed under a short-wave UV lamp. The R_F values for ATP, ADP, and AMP were 0.16, 0.32, and 0.54, respectively. For quantitation directly on the plates a dual-beam spectrodensitometer (Schoeffel Instruments, Model SD 3000), with monochomator (Model QPM 30), density computer (Model SDC 300), and integrating strip recorder (Model SDR 303) was used. This instrument combination uses a dual-beam ratio-type system which minimizes errors due to changes in adsorbent layer thickness and a monochromator which allows a

wavelength selection of 200–700 mμ. Optical density values are recorded continually, and areas under absorption peaks are integrated and recorded separately. The sensitivity of the system can be increased by setting the recorder full scale at lower optical density values. In our study, a sensitivity of 0.4 optical density units full scale was used. Each strip of the developed chromatogram was scanned at a wavelength of 260 mμ, the wavelength of maximum absorption for adenine nucleotides. The scanning of one strip requires *ca.* 60 sec. The areas under the resulting absorption peaks were obtained both from the integrating strip recorder (arbitrary counts) and by triangulation (area = height × width at 1/2 height). The correlation between the results of the two methods of integration was excellent. The reference curves for all three nucleotides were identical; thus, only one curve was necessary. This curve is illustrated in Fig. 1. Each point on this curve represents the mean of ten determinations. The probable error was *ca.* 2–3% at quantities over 5 mμmoles and *ca.* 5% at quantities under 5 mμmoles. As little as 0.5 mμmoles of the adenine nucleotides could be detected with fair precision. Reference curves for ATP, ADP, and AMP were also obtained by spotting, separating and scanning mixtures of these compounds in known concentrations. The resulting peaks were nearly always com-

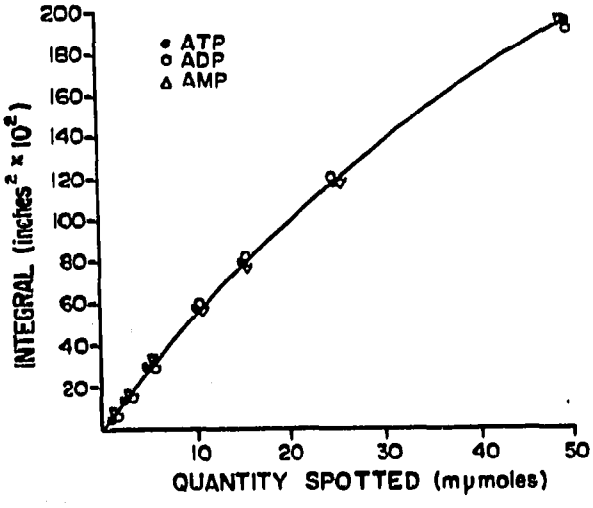

Fig. 1. Reference curve for adenine nucleotides. Each point represents the mean of ten determinations.

pletely separated. However, in some cases the optical density read-out did not return to baseline between absorption peaks. In such cases the integral obtained from the integrating strip recorder was obviously in error, and the area computed by triangulation was used solely. The reference curves so obtained were essentially identical to that shown in Fig. 1.

For chromatography of the myocardial adenine nucleotides, an aliquot of each myocardial extract (5–10 μl) was spotted on a separate strip of a scored plate as described above. The reference solutions for the three adenine nucleotides were also spotted on the same plate. As many as six myocardial samples and the three reference solutions could be spotted on each plate. The chromatogram was then developed in distilled water for a distance of *ca.* 12–14 cm. Since the migration of basic or neutral compounds (nucleosides and nucleobases) on PEI-impregnated cellulose is deter-

mined by partition principles, these compounds migrate on the cellulose layer with water development. However, the migration of the acidic compounds (nucleotides) on this absorbent depends on the principle of anion exchange. These compounds, consequently, remain at the origin during the water development. This initial development, therefore, serves to separate the nucleosides and nucleobases from the

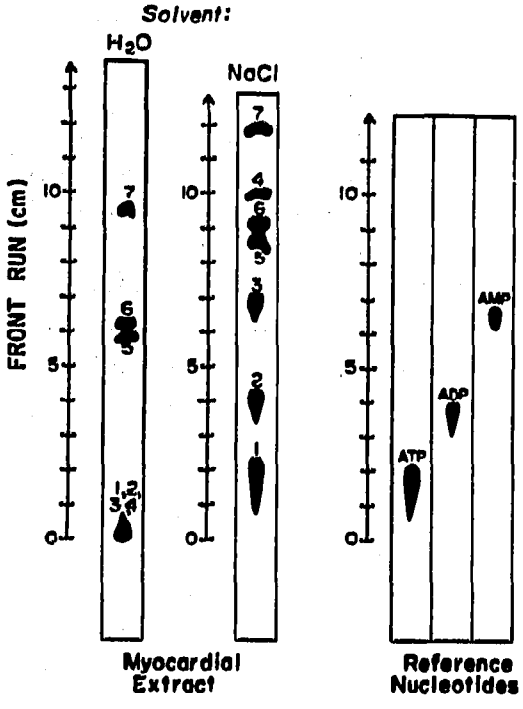

Fig. 2. Tracing of chromatogram showing migrations of myocardial ATP (spot 1), ADP (spot 2), and AMP (spot 3) after development in water and sodium chloride. Migrations of reference adenine nucleotides in sodium chloride are also shown. Other myocardial purines tentatively identified are IMP (spot 4), adenosine (spot 5), hypoxanthine (spot 6) and inosine (spot 7).

nucleotides. Following the first development, the chromatogram was air dried and developed in 1.25 *M* NaCl as described above. Each development requires *ca.* 45 min. The migrations of the adenine nucleotides following the first and the second development are illustrated in Fig. 2, which shows a chromatogram of reference nucleotides and a chromatogram of myocardial extract after 10-min arrest. A detailed description of the behavior of nucleotides, nucleosides, and nucleobases on PEI impregnated cellulose thin layers has been given by RANDERATH AND RANDERATH[1]. Following the sodium chloride development, three well defined absorption peaks with R_F values identical to the reference nucleotides were consistently observed. These absorption peaks were tentatively identified as ATP, ADP, and AMP. The chromatogram was then scanned at a wavelenth of 260 mμ, and the resulting absorption peaks were integrated as described. In many cases the identity of the spots tentatively identified as ATP, ADP, and AMP were confirmed by scanning the chromatogram at wavelengths from 230–290 mμ in 5-mμ increments and comparing the absorbance–wavelength relationships to those obtained using the reference ATP, ADP, and AMP. In all cases this relationship was identical for the reference and the respective myocardial nucleotide. The identities of the adenine nucleotides in the myocardial ex-

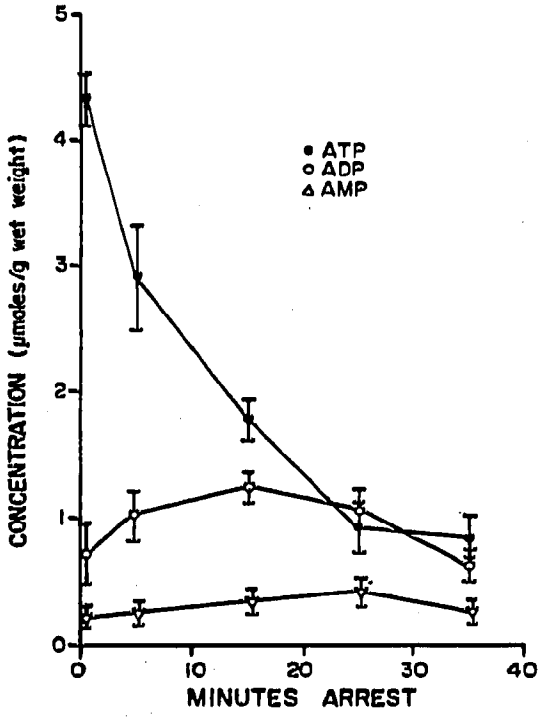

Fig. 3. Changes in myocardial concentrations of acid-soluble ATP, ADP, and AMP during cardiac arrest.

tracts was further verified by spotting larger volumes of extract (20–40 μl) and spraying the developed chromatogram with molybdic acid reagent. Dark blue spots at the positions of ATP, ADP, and AMP indicated the presence of sugar phosphates. One other nucleotide consistently identified in the myocardial extracts during cardiac arrest was inosine monophosphate (IMP). Adenosine, inosine, and hypoxanthine were also tentatively identified during arrest. No systematic study of these compounds has been undertaken at this time.

Results and discussion

The changes in the myocardial concentrations of acid-soluble adenine nucleotides during cardiac arrest are shown in Fig. 3. These data are mean values collected from five dog hearts by the procedure described. The control concentrations of ATP, ADP, and AMP averaged 4.35, 0.71, and 0.21 μmoles/g wet weight, respectively. The control ATP:ADP ratio was 6.1 and the control ATP:AMP ratio was 20.7. During arrest the concentration of ATP declined rapidly, and after 35 min was only 0.85 μmoles/g wet weight. Coincident with the reduction in ATP concentration, the concentrations of ADP and AMP rose initially but subsequently declined.

The results obtained in the present study by use of anion-exchange thin-layer chromatography agree well with myocardial free adenine nucleotide concentrations reported by others using different techniques. Table I compares our control results with those of other investigators. Furthermore, our results during cardiac arrest compare excellently with anoxic heart studies by others. FEINSTEIN[7], BENSON *et al.*[4], and IMAI *et al.*[5] have also noted a rapid reduction in myocardial ATP with a concomitant rise in ADP and AMP during myocardial anoxia. Thus, it is evident that the

TABLE I

ACID-SOLUBLE ADENINE NUCLEOTIDE CONCENTRATIONS IN NORMAL MAMMALIAN HEARTS: A COMPARISON OF RESULTS

Investigator	*ATP*[a]	*ATP:ADP*	*ATP:AMP*	*Animal*	*Methods*
BOERTH *et al.*[2]	5.89	—	—	dog	Firefly luminescence
LEFER *et al.*[3]	3.65	—	—	dog	Firefly luminescence
BENSON *et al.*[4]	4.30	3.3	8.6	dog	Separation by column chromatography on ion-exchange resin; quantitation by UV absorption
IMAI *et al.*[5]	4.04	6.0	23.8	rabbit	ATP by firefly luminescence; ADP and AMP by firefly luminescence after enzymatic conversion to ATP
RICHMAN AND WYBORNY[6]	4.10	10.0	20.0	rabbit	Separation by paper chromatography; quantitation by UV absorption after elution from paper
FEINSTEIN[7]	5.59	8.7	16.4	guinea-pig	Enzymatic spectrophotometric analysis
Present study	4.35	6.1	20.7	dog	Separation by anion-exchange thin-layer chromatography with quantitation directly on plates by UV absorption

[a] Expressed as micromoles/g wet weight.

technique described here is at least as reliable as the currently employed methods for determination of tissue acid-soluble adenine nucleotides. In addition, we believe the determination of these compounds on anion-exchange thin layers offers the advantage of speed and simplicity.

This work was supported by NIH grant No. HE-11780.

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Received August 19th, 1971