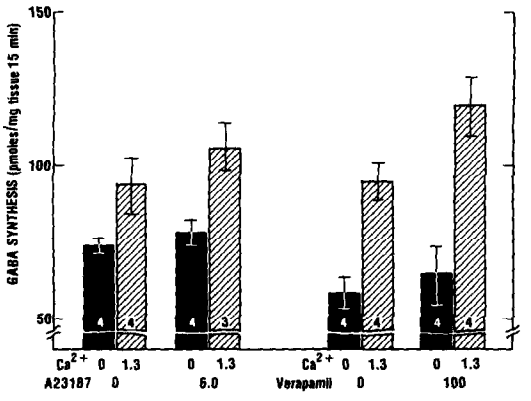

Fig. 3. Effect of A23187 and verapamil on calcium-stimulated GAD activity in striatal slices. Each value is the mean ± S.E.M. of the number of samples indicated in the figure. A23187 was dissolved in absolute ethanol and then added to the incubation. Ethanol was included in the control incubations. Ca^{2+} concentrations are mM; A23187 and verapamil concentrations are μM.

concentration-related and could be blocked by La^{3+}, but not by Co^{2+} or by verapamil at the concentrations tested. Submaximal stimulation by Ca^{2+} was not potentiated by the ionophore A23187. These data are consistent with the hypothesis that extracellular Ca^{2+} plays a role in the regulation of GABA biosynthesis by interaction with binding sites on the exterior surface of neuronal membranes. The mechanism by which Ca^{2+} is transduced to stimulate GAD activity cannot be explained by these data.

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Isolation and separation of heme *a* and heme *b* from cardiac tissue by thin-layer chromatography

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Hemoproteins found in mammalian cells are composed of a protein moiety coupled with either heme *a*, *b*, or *c*. Hemes *a* and *b* are loosely associated with the protein moiety of hemoproteins and are extractable under acid conditions into certain organic solvents [1]. Heme *c* is covalently linked to the protein moiety of hemoproteins and special procedures are required to break the covalent bond prior to heme *c* isolation [2].

The isolation of heme *b* from tissues is facilitated by the isolation of subcellular fractions (i.e. microsomal fractions) which are essentially free of heme *a*. The isolation of heme *a* from tissue is complicated by the fact that heme *a* is primarily located in the mitochondria, which also contains heme *b*.

Most published procedures for the isolation of heme *a* are relatively complex [3–7] and utilize a large amount of tissue for heme *a* isolation. This paper will describe a simple method for the isolation of heme *a* and heme *b* from small quantities of cardiac tissue.

Materials and methods

Hemin (Type III) was purchased from the Sigma Chemical Co. (St. Louis, MO). Silica gel 7 was obtained from the J. T. Baker Co. (Phillipsburg, NJ). Polyvinyl alcohol (100% hydrolyzed, average molecular weight of 14,000) was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Aquasol-2 and [4-^{14}C]-δ-aminolevulinic acid (25.4 mCi/mmole) were purchased from the New England

Nuclear Corp. (Boston, MA).

Preparations of thin-layer plates. Polyvinyl alcohol (7.5 g) was dissolved in 160 ml water by heating at 90°. A silica gel slurry was prepared by mixing 50 g of silica gel 7 with the heated polyvinyl alcohol solution in a Waring blender. The silica gel slurry was then degassed under a water aspirator vacuum. The thin-layer plates were prepared at a thickness of 300 μm using a Camag plate coating applicator.

Isolation of hemes. Male Sprague–Dawley rats (200–225 g) were allowed food and water *ad lib.* The animals were killed by cerebral concussion, and the hearts were excised and immediately perfused with iced 0.9% NaCl by cannulation of the aorta. All of the following procedures were performed at 4°.

Cardiac homogenates (33.3%, w/v) were prepared in 0.1 M phosphate buffer (pH 7.4) using 20 sec bursts of a Polytron homogenizer. The homogenate (1.5 ml) was extracted with 15 ml ether for 5 min and centrifuged at 2000 *g* for 5 min. The ether layer was discarded. The aqueous layer and pellet were extracted twice with 7.5 ml of acid acetone (1.8%, v/v conc. HCl in acetone). The supernatant fractions were combined with 15 ml ether and 7.5 ml KCl (5%, w/v) and were shaken for 15 sec. The organic phase was then dried under oxygen-free nitrogen until approximately 0.5 ml remained. Chloroform–methanol (2:1, 0.2 ml) was added, and two phases were identifiable after centrifugation at 1000 *g* for 2 min. The lower phase containing the hemes was streaked onto the silica gel using a Camag Chromatocharger. The silica gel plates were developed in absolute ethanol–water–formic acid (80:20:2) for approximately 3 hr. The silica gel containing the hemes was scraped from the plate and the hemes were extracted from the silica gel with 5 ml of 1.8% (v/v) concentrated HCl–acetone. The acid acetone solution was taken to dryness under nitrogen. Heme *b* was identified and quantified from its pyridine hemochromogen spectrum using an Aminco DW-2 spectrophotometer by the method of Porra and Jones [8]. Heme *a* was identified from its pyridine hemochromogen spectrum using an Aminco DW-2 spectrophotometer by the method of Vanderkooi and Stotz [9]. For determination of radioactivity, the hemes were dissolved in 0.2 ml methanol, Aquasol-2 was added, and radioactivity was determined by standard liquid scintillation technology.

Preparation of [^{14}C]*heme* a *and* [^{14}C]*heme* b. Rats were injected with 20 μCi of [4-^{14}C]-δ-aminolevulinic acid 24 and 48 hr prior to their being killed. The livers were perfused *in situ* with iced 0.9% NaCl, and hemes *a* and *b* were isolated and separated by the procedure described previously. The hemes were dissolved in 0.1 N NaOH, and then 0.1 M phosphate buffer (pH 7.4) was added. After adjusting the pH to 7.4, aliquots of the [^{14}C]hemes were added to cardiac homogenates for determination of heme recovery.

Results

The separation of hemes *a* and *b* from cardiac tissue was accomplished on silica gel 7–polyvinyl alcohol plates. The R_f values for hemes *a* and *b* were 0.90 and 0.75 respectively. The identity of heme *a* was established by spectral characteristics of the heme *a* pyridine hemochromogen, which was similar to those described by Morrison *et al.* [10], Vanderkooi and Stotz [9], and Yoshida and Shimazono [6]. The displacement of the spectrum caused by carbon monoxide binding was also in agreement with that described by Vanderkooi and Stotz [9]. Heme *b* was identified by the spectral characteristics of its heme *b* pyridine hemochromogen.

The recovery of heme *b* from cardiac homogenates is shown in Table 1. As determined by the pyridine hemochromes, 70% of the heme *b* content in the heart was recovered in the acid acetone extract and approximately 45% of the heme *b* applied to the thin-layer plate was

Table 1. Recovery of heme *b* by thin-layer chromatography*

Source	Experiment 1	Experiment 2
	Recovery of [^{14}C]heme *b*† (dpm)	
Cardiac homogenate	28,774	45,637
Acid acetone extract	22,979	35,225
TLC plate	9,694	14,813
	Recovery of heme *b* as determined by pyridine hemochromogen‡ (nmoles)	
Cardiac homogenate	96	122
Acid acetone extract	69	80
TLC plate	30	38

* Each value is the mean of two samples.

† Heme *b* as isolated from liver was added to a 33.3% (w/v) homogenate of rat heart in phosphate buffer (0.1 M, pH 7.4).

‡ Ref. 8.

Table 2. Recovery of heme *a* by thin-layer chromatography

Source	Recovery of [^{14}C]heme *a** (dpm) Experiment 1	Experiment 2
Cardiac homogenates	9569	9906
Acid acetone extract	6423	4795
TLC plate	2489	1876

* Each value is the mean of two samples. Heme *a* as isolated from liver was added to a 33.3% (w/v) homogenate of rat heart in phosphate buffer (0.1 M, pH 7.4).

recovered. The recovery of heme *b* determined by measuring [^{14}C]heme *b* was in good agreement with the recovery of heme *b* as determined by the pyridine hemochromogen method. The recovery of heme *a* is shown in Table 2. Approximately 55% of added heme *a* radioactivity was recovered in the acid acetone extract and approximately 40% of the heme *a* applied to the thin-layer plate was recovered. Essentially no heme *b* was found to contaminate heme *a* isolated from the thin-layer plate and only a small amount of heme *a* contamination was found in heme *b* isolated from the thin-layer plate (Table 3).

Glycine, δ-aminolevulinic acid, and uroporphyrins were not retained in the organic phase spotted on the thin-layer plate, and protoporphyrin IX, which was extracted from cardiac tissue, remained near the origin on the thin-layer plate in this system.

Table 3. Separation of heme *a* and heme *b*

Recovery of heme *b* in heme *a**	
Heme *b* added to cardiac homogenate	104,630 dpm
Heme *b* recovered in heme *a* fraction	732 dpm
% Contamination	<1%
Recovery of heme *a* in heme *b*†	
Heme *a* added to cardiac homogenate	9,569 dpm
Heme *a* recovered in heme *b* fraction	146 dpm
% Contamination	1.5%

* Radioactive heme *b* was added to cardiac homogenates and heme *a* was isolated as described in Materials and Methods.

† Radioactive heme *a* was added to cardiac homogenates and heme *b* was isolated as described in Materials and Methods.

Discussion

The investigation of the properties of cellular hemes, as well as the mechanisms of their synthesis and breakdown, requires the development of techniques to isolate these hemes. This report describes a technique for simultaneous isolation of hemes *a* and *b* from small amounts of cardiac tissue by employing a standard extraction procedure coupled with a thin-layer chromatography system using silica gel 7 in conjunction with a polyvinyl alcohol binder. The polyvinyl alcohol did not serve merely as a binder but also as an integral ingredient in the separation procedure. All commercially available silica gel thin-layer plates that were tested were not effective and could not be used for these separations. Commercial plates do not have polyvinyl alcohol as a binder. The utilization of this procedure for measuring the separate rates of synthesis and catabolism of hemes *a* and *b* in biological systems is implicit, and studies which have been devised to test the effects of perturbants on the synthesis and catabolism of hemes *a* and *b* will be the subject of a future report.

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Antiplatelet actions of trimetoquinol isomers: evidence for inhibition of a prostaglandin-independent pathway of platelet aggregation

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Trimetoquinol (TMQ) possesses both β-adrenoreceptor stimulant [1, 2] and antiplatelet activities [3, 4]. TMQ exists as two stereoisomers and it has been reported that the β-stimulant activity resides primarily with *S*(−)-TMQ [1, 5, 6], whereas antiplatelet activity is selectively exhibited by the *R*(+)-isomer [3, 7]. Our findings that *R*(+)-TMQ is more effective than *S*(−)-TMQ as an inhibitor of platelet aggregation induced by arachidonic acid, thromboxane A_2 and U46619 (a stable PGH_2 analog) suggest that TMQ stereoselectively inhibited the prostaglandin-dependent pathway of platelet activation [7]. Since *R*(+)-TMQ inhibits prostaglandin-mediated platelet aggregation [7] and is considerably less active than *S*(−)-TMQ as an agonist of the bronchodilator activity [2, 6], it is likely that the pharmacological activities of the TMQ isomers are mediated by different mechanisms. To further examine the antiplatelet