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HEME BIOSYNTHESIS IN THE HEART

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Abstract—The rates of biosynthesis of heme *a* and heme *b* in hearts of fed and fasted rats were measured using an isolated heart perfusion system. δ-Aminolevulinic acid synthetase activity was decreased in hearts of fasted rats to about 30% of values in hearts obtained from fed rats. [^{14}C]Glycine incorporation into hemes *a* and *b* of cardiac tissue obtained from fasted rats was also decreased to about 30% of values obtained in hearts from fed rats. Cobalt addition to the perfusion fluid led to a decrease in cardiac δ-aminolevulinic acid synthetase activity just as cobalt administration to rats does *in vivo*. These studies strongly suggest that δ-aminolevulinic acid synthetase activity regulates the rate of synthesis of hemes *a* and *b* in the heart.

It is generally presumed that heme is synthesized in animal cells from glycine and succinyl-CoA by a common enzymatic pathway [1–4] and that the rate of heme biosynthesis is regulated in the cell to meet the requirements of hemoprotein synthesis and to ensure that excess heme or heme precursors do not accumulate under normal conditions. Studies in the liver which have demonstrated that δ-aminolevulinic acid (ALA) synthetase is the rate-limiting enzyme in hepatic heme biosynthesis [5–9] indicate that this enzyme assumes a principal role of regulating heme biosynthesis in all tissues. The regulation of ALA synthetase activity and its role in regulating heme biosynthesis have not been studied thoroughly in extrahepatic organs [10–16]. Recent reports have indicated that the mechanism of regulation of cardiac ALA synthetase activity may be different than that described for the hepatic enzyme [11, 12, 14, 16]. Cardiac ALA synthetase activity was not decreased by the administration of heme [16], nor was it increased by allylisopropylacetamide or 3,5-dicarbethoxy-1,4-dihydrocollidine [11], agents which markedly increase the activity of the hepatic enzyme [3]. Fasting of rats for 18 or more hours leads to cardiac ALA synthetase activity that is markedly reduced from control values found in fed animals [16]. The activity of the hepatic enzyme remains relatively unchanged in fasted rats [10]. Recent observations [12] show that cardiac ALA synthetase activity increases in rats subjected to rigorous exercise.

ALA synthetase activity as determined *in vitro* may provide an index of heme biosynthesis but its activity is only an indirect measure of the capacity of synthesis in a given organ. A better and more direct method of establishing the rate of heme biosynthesis is by measuring the rate of incorporation of heme precursors, especially glycine, into heme. These studies have been difficult because only a small fraction of the total free glycine in the cell is incorporated into heme [17], and the rate of heme synthesis derived by this method is dependent on the concentration of free glycine and the rate of uptake of labeled glycine into the cell. Previous studies which have employed this method have generally not distinguished among the possibilities that changes in glycine concentration, in glycine uptake, or in the activity of enzymes in the pathway could account for changes in the rate of heme biosynthesis observed in tissue [18, 19]. An exception is the work of Cowtan *et al.* [20] who demonstrated a regulation of hepatic heme synthesis by glycine.

This report presents studies on isolated perfused rat heart preparations which were employed: (1) to demonstrate that the heart synthesizes heme from precursors that have been utilized in other studies concerned with other organs; (2) to demonstrate that heme *a* as well as heme *b* synthesis is controlled by the rate of protoheme synthesis; (3) to determine the rate of cardiac heme biosynthesis using [^{14}C]-glycine as a precursor for cardiac heme; and (4) to demonstrate that ALA synthetase activity is probably the rate-limiting enzyme in cardiac heme biosynthesis in the heart.

MATERIALS AND METHODS

Bovine serum albumin (fraction 5) and insulin (bovine) were purchased from the Sigma Chemical Co. (St. Louis, MO). [2-^{14}C]Glycine (14.1 mCi/mmole) and [4-^{14}C]-δ-aminolevulinic acid hydrochloride (25.4 mCi/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). Unisol and Unisol Complement were obtained from Isolab, Inc. (Akron, OH).

Initiation of perfusion. The male Sprague–Dawley rats (200–250 g) used in these studies were allowed food (Purina Chow 5008) and water *ad lib.* or were fasted 24 hr prior to being killed. The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and heparin (600 units) was administered via the tail vein. The hearts were then excised, chilled in iced 0.9% NaCl, and perfused by the Langendorff

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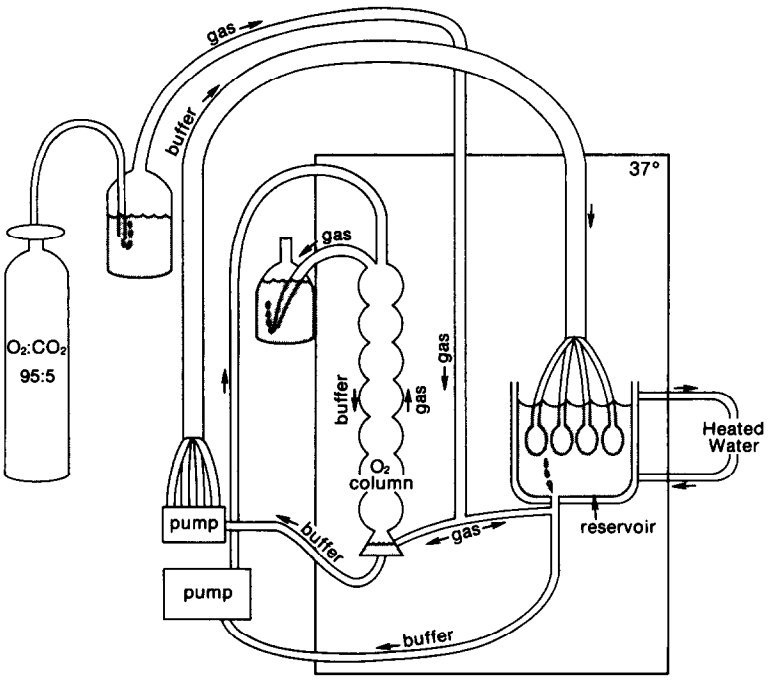

Fig. 1. The apparatus employed for isolated heart perfusion.

technique* as described by Morgan *et al.* [21]. The perfusion was initiated when a cannula was inserted and tied in the aorta.

The first 10 ml of perfusion medium that passed through the heart, which washed out all visible blood, was discarded. Ten minutes were allowed to elapse after the heart was cannulated prior to the initiation of an experiment to allow the hearts to recover from the anoxia associated with the processes of excision and cannulation.

Perfusion medium. A modified Krebs–Henseleit bicarbonate buffer [22], which contained plasma levels of amino acids, 15 mM glucose, 25 mU/ml insulin and 1% albumin at 37°, pH 7.4, and was equilibrated with O_2:CO_2 (95:5), was employed in all experiments. The volume of medium that was recirculated was 150 ml. In certain experiments, [^{14}C]glycine (200 μCi for 1-hr perfusions, 100 μCi for 2- and 3-hr perfusions) or [^{14}C]ALA (25 μCi) was added to the perfusion medium at the initiation of an experiment. Rates of incorporation were determined at linearity. In other experiments, cobaltous chloride·$6H_2O$ at the indicated concentration of cobalt in excess of the EDTA concentration was added to the perfusion medium at the initiation of an experiment.

Perfusion apparatus. The apparatus diagrammed in Fig. 1 was employed to maintain four hearts in each experiment. The hearts were suspended in the perfusion chamber which also served as a reservoir for the perfusion medium. A Cole–Parmer Masterflex pump delivered the perfusion medium from the base of the perfusion chamber to the top of the oxygenation column. A Gilson Minipulse II peristaltic pump delivered the perfusion medium from the base of the oxygenation column to each heart through individual cannulae assemblies at a rate of 7.2 ml/min which generated a mean perfusion pressure of 45 mm Hg.

A mixture of O_2:CO_2 (95:5) was slowly bubbled through the medium in the perfusion chamber and flowed up through the oxygenation column. Heated water was pumped through the water jacket surrounding the perfusion chamber to maintain the perfusion medium at 37°.

Termination of a perfusion. At the indicated time after the initiation of an experiment, the perfusions were terminated in the following manner: (1) the hearts were placed in iced 0.9% NaCl if ALA synthetase activity was determined; (2) the hearts were frozen with Wollenberger clamps while still being perfused if ATP and creatine phosphate levels were determined; (3) the hearts were perfused for 4 min with iced 0.9% NaCl which contained 1.3 mM alanine if [^{14}C]glycine was employed in the experiment; and (4) the hearts were perfused for 4 min with iced 0.9% NaCl if [^{14}C]ALA was employed in the experiment.

Analytical procedures. Cardiac ALA synthetase activity was determined by a modification of the method of Briggs *et al.* [11] as described earlier [16]. ATP and creatine phosphate levels were determined by the method of Lowry and Passonneau [23]. Radioactivity in cardiac heme *a* and heme *b* was determined by a thin-layer chromatographic method [24]. Heme *b* content was determined by the method of Porra and Jones [25]. Glycine radioactivity and glycine content in the isolated perfused hearts were determined by a modification of the method of Ohmori *et al.* [26] which is based on the stoichiometric conversion of glycine to hippurate acid which is then isolated and quantified.

* O. Langendorff, *Pflügers Arch. geo. Physiol.* **61**, 291 (1895).

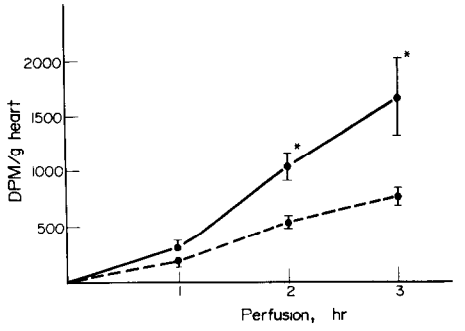

Fig. 2. Incorporation of [^{14}C]glycine into cardiac heme *b* in the isolated perfused rat heart. Heme *b* was isolated as described in Ref. 23. The solid line denotes hearts from fed rats, and the dashed line indicates hearts from fasted rats. An asterisk (*) denotes a significant increase from fasted values ($P < 0.05$). Each point is the mean of at least five hearts ± S.E.

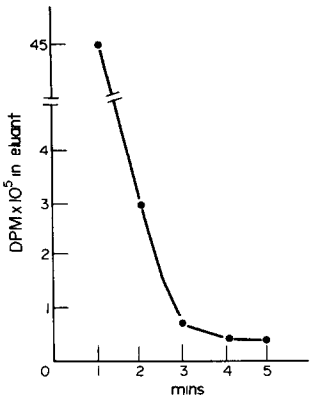

Fig. 4. [^{14}C]Glycine washout from the isolated perfused rat heart. At the termination of perfusion (zero time), the hearts were perfused with iced 0.9% NaCl which contained 1.3 mM alanine. Each point is the mean value determined from four hearts; the total volume of eluant was 150 ml.

Cobalt levels were determined in rats injected subcutaneously with 60 mg/kg of $CoCl_2 \cdot 6H_2O$. The serum was diluted with water and then analyzed on a Perkin–Elmer model 360 atomic absorption spectrophotometer using an air–acetylene flame at 240.7 nm.

Radioactivity in the heart was determined by solubilizing 0.1 ml or a 20% homogenate of cardiac tissue in 5% KCl (w/v) with 1.0 ml of Unisol overnight. Methanol (0.5 ml) and Unisol Complement (10 ml) were then added, and radioactivity was determined by standard liquid scintillation counting techniques.

RESULTS

Heme biosynthesis in the heart. [^{14}C]Glycine was incorporated into both cardiac heme *a* and heme *b* in the isolated perfused rat hearts (Figs. 2 and 3). However, the level of [^{14}C]glycine incorporated into hemes *a* and *b* was markedly reduced in the perfused hearts of fasted rats. Although the level of [^{14}C]-glycine incorporation into hemes is expressed as dpm/g of heart, similar data were obtained when expressed as dpm/mole of heme.

Since it was possible that the decline of [^{14}C]-glycine incorporation into cardiac hemes *a* and *b* may have resulted from a difference in the concentration or specific activity of glycine in hearts of fasted rats, the level of free glycine and [^{14}C]glycine was determined in the perfused hearts. Before this determination could be made, it was important to flush interfering extracellular glycine from the hearts. The perfusion was terminated by perfusing the hearts with iced 0.9% NaCl which contained 1.3 mM alanine (added to compete with glycine for membrane transport) [27]. A time period of 4 min was selected for the length of the washout since the radioactivity in the washout solution did not decrease during the next minute of perfusion; the radioactivity collected during min 3 was less than 0.5% of that collected during min 1 and the counts collected during min 3 of washout were less than 5% of the counts detected in the hearts (Fig. 4).

The levels of [^{14}C]glycine and free glycine in hearts after glycine washout are shown in Figs. 5 and 6. A small difference between the levels of [^{14}C]glycine in the hearts of the fed and fasted rats was observed after 1 hr of perfusion, but no differences were

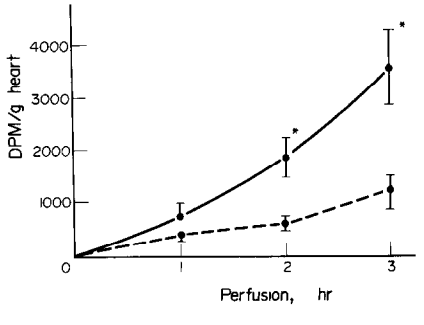

Fig. 3. Incorporation of [^{14}C]glycine into cardiac heme *a* in the isolated perfused rat heart. Heme *a* was isolated as described in Ref. 23. The solid line denotes hearts from fed rats, and the dashed line indicates hearts from fasted rats. An asterisk (*) denotes a significant increase from fasted values ($P < 0.05$). Each point is the mean of at least five hearts ± S.E.

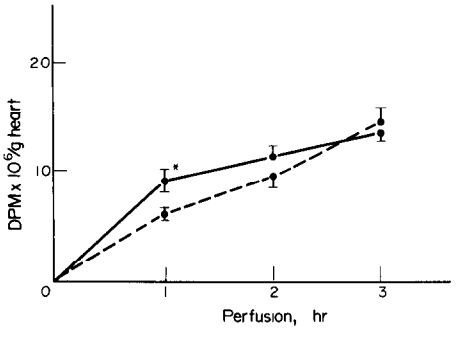

Fig. 5. [^{14}C]Glycine content in the isolated perfused rat heart. [^{14}C]Glycine content was determined as described in Materials and Methods. The solid line denotes hearts from fed rats and the dashed line indicates hearts from fasted rats. An asterisk (*) denotes a significant increase from fasted values ($P < 0.05$). Each point is the mean of at least five hearts ± S.E.

Table 1. [^{14}C]ALA incorporation into cardiac heme *a* and heme *b* in isolated perfused rat heart

	Homogenate (dpm/g heart)	Heme *b** (dpm/100 μmoles heme *b*)	Heme *a** (dpm/g heart)
Fed	5940 ± 1420	4500 ± 1040	1640 ± 130
Fasted†	6440 ± 2660	4800 ± 1620	1250 ± 320

* Each value is the group mean ± S.E.M. of four hearts. The hearts were perfused with [^{14}C]ALA for 2 hr. Hemes *a* and *b* were isolated as described in Materials and Methods.

† Rats were fasted for 24 hr.

detected in hearts perfused for 2 or 3 hr (Fig. 5). No difference in the contents of free glycine in the hearts of the fed and fasted rats was observed during the course of the perfusion (Fig. 6).

The levels of [^{14}C]ALA incorporation into hemes *a* and *b* in hearts perfused for 2 hr are shown in Table 1. No difference was observed between the levels of incoporation in hearts obtained from fed and fasted rats. Since a difference in [^{14}C]ALA uptake could mask a difference in the rate of [^{14}C]ALA incorporation into heme caused by changes in the activity of enzymes in the pathway, the levels of radioactivity in the hearts were determined after [^{14}C]ALA was flushed from the hearts. A washout experiment essentially identical to that described for glycine indicated that interfering [^{14}C]ALA was flushed from the hearts after 4 min. The mean levels of radioactivity in hearts from fed and fasted rats were not different.

Since a decrease in the amount of [^{14}C]ALA incorporated in cardiac hemes *a* and *b* was not detected in hearts obtained from fasted rats, a decrease in ALA synthetase activity was most likely responsible for the decrease in [^{14}C]glycine incorporation into the heme of hearts obtained from fasted rats. The rate of [^{14}C]glycine incoporation was reduced to about one-third in fasted rats and ALA synthetase activity was reduced to about one-third in hearts obtained from fasted rats (Fig. 7).

Viability of the perfused hearts and suitability of the perfused hearts for studying cardiac heme biosynthesis. Several criteria were used to judge the viability of the perfused rat hearts. The hearts maintained a regular heart rate which ranged from 200 to 225 beats/min once they had recovered from the trauma of the cannulation procedure. Any heart which failed to beat was rejected from the studies. The heart became noticeably edematous only after the second hour of the perfusion, at the perfusion pressure employed in the experiments.

The levels of ATP and creatine phsophate were also determined, to judge the viability of the perfused heart. Although there was some general decline in the levels of these high energy compounds from the levels detected *in vivo* (Table 2), the levels of these compounds remained much higher than those reported when ischemia or anoxia was occurring in the preparation [28].

The levels of ALA synthetase activity in the perfused hearts were unchanged or slightly increased, which also indicates that the hearts were in good condition (Fig. 7). ALA synthetase activity declined to 6.5 ± 0.5 nmoles·(g heart)$^{-1}$·hr^{-1} in hearts that were not cannulated and were immersed in the perfusion buffer for 2 hr at 37°.

Serum was obtained from rats treated with cobaltous chloride (60 mg/kg, i.p.); and the levels of cobalt at 30 and 60 min after treatment were 19 and 21 ppm respectively. The addition of cobalt to the perfusion medium at a concentration of 21 or 10.5 ppm caused a marked decrease in ALA synthetase activity in the hearts obtained from fed rats after 1 hr of perfusion (Table 3). This was similar to the decrease of ALA synthetase activity observed *in vivo* 1 hr after treatment [16].

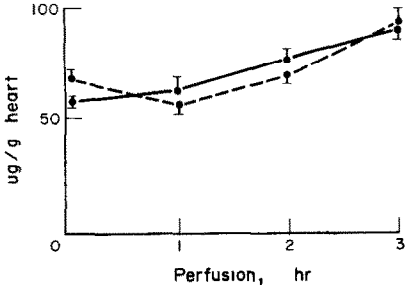

Fig. 6. Free glycine content of isolated perfused rat hearts. The solid line denotes hearts from fed rats, and the dashed line represents data obtained from fasted rats. Each point is the mean value of at least five hearts ± S.E.

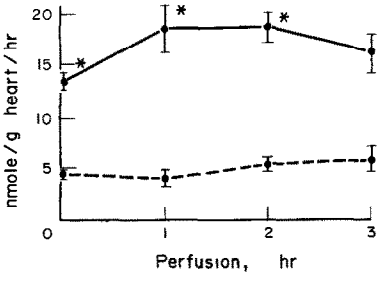

Fig. 7. ALA synthetase activity in the isolated perfused rat heart. ALA synthetase activity was determined in cardiac homogenates, as described in Materials and Methods. The solid line denotes hearts from fed rats, and the dashed line represents data from fasted rats. An asterisk (*) denotes a significant increase from fasted values ($P < 0.05$). Each point is the mean of at least five hearts ± S.E.

Table 2. ATP and creatine phosphate levels in isolated perfused hearts from fed and fasted rats

Treatment	ATP* (μmoles/g dry weight)		Creatine phosphate* (μmoles/g dry weight)	
	Fed	Fasted†	Fed	Fasted†
Control	18.0 ± 1.0	22.5 ± 1.5	18.5 ± 1.5	17.5 ± 2.0
1-hr Perfusion	14.0 ± 1.5	18.5 ± 1.5‡	14.5 ± 1.5‡	26.5 ± 3.0‡
2-hr Perfusion	15.5 ± 4.0	15.5 ± 2.5‡	15.0 ± 3.5	13.0 ± 2.5

* Each value is the group mean ± S.E.M. of at least five animals. ATP and creatine phosphate levels were determined as described in Materials and Methods. Control values represent the *in vivo* level obtained on freeze-clamped hearts.

† Rats were fasted for 24 hr.

‡ Denotes significant differences from control ($P < 0.05$).

DISCUSSION

This report describes an isolated perfused rat heart system which facilitates the study of the regulation of cardiac ALA synthetase and cardiac heme biosynthesis. The perfused hearts are viable, and the ALA synthetase activity is regulated in the perfused hearts by cobalt in a manner similar to that observed *in vivo*. Viability of a perfused heart was indicated by the maintenance of a regular heart rate and the retention of high levels of ATP, creatine phosphate, and ALA synthetase activities in the hearts. The preservation of cardiac ALA synthetase activity in the perfused heart is important because of its impact on potential studies of cardiac heme synthesis in addition to its being, apparently, a good indicator of heart condition, since ALA synthetase activity declined in the fasting state as well as in unperfused hearts.

It was demonstrated that [^{14}C]glycine and [^{14}C]-ALA were incorporated into cardiac heme, thus establishing that the heart is capable of synthesizing heme from precursors utilized by the liver. The decreased rate of incorporation of [^{14}C]glycine into cardiac hemes *a* and *b* of fasted rats coincided well with the decreased activity of cardiac ALA synthetase. This indicates that the ALA synthetase activity was probably regulating the rate of cardiac heme biosynthesis. These data could be explained by a reduction in glycine concentration or a decrease in the specific activity of glycine in perfused hearts obtained from fasted rats, but no differences were observed between the contents of perfused hearts of fed and fasted rats. Only a small difference was observed in [^{14}C]glycine uptake. Therefore, the difference in the incorporation of glycine into cardiac hemes *a* and *b* in perfused hearts from fed and fasted rats cannot be accounted for by differences in the concentration of glycine or the uptake of [^{14}C]glycine. It should be noted that in previous studies which have used the incorporation of radioactive glycine into hemes as an index of the rate of heme biosynthesis no data were reported on the concentration of glycine or the level of radioactive glycine in the tissue [18–19]. Finally, cobalt addition to heart perfusates leads to decreased cardiac ALA synthetase activity such as is seen when rats are treated with cobaltous chloride *in vivo* [16].

Although the decrease in [^{14}C]glycine incorporation into cardiac hemes appeared to be related to the decline of ALA synthetase activity in perfused hearts of fasted rats, a decrease of the activity of other enzymes in the heme biosynthetic pathway could have been responsible for the decreased rate of heme synthesis. However, no difference in the amount of [^{14}C]ALA incorporated in cardiac hemes *a* or *b* was observed in perfused hearts of fed or fasted rats. This suggests that steps beyond ALA formation are not altered in hearts obtained from fasted rats. Therefore, ALA synthetase activity limits the rate of cardiac heme synthesis in the fasted rat and, in all probability, in the fed rat.

Table 3. Effect of cobaltous chloride on cardiac ALA synthetase activity in the isolated perfused rat heart

Treatment	ALA synthetase activity* [mmoles·(g heart)$^{-1}$·hr^{-1}]
Fed control	10.0 ± 1.0
Fasted control†	4.0 ± 0.5
Cobalt (21 ppm) in the perfusate‡	6.0 ± 0.5§
Cobalt (10.5 ppm) in the perfusate‡	7.5 ± 1.0§

* Each value is the group mean ± S.E.M. of four animals. ALA synthetase activity was determined in cardiac homogenates as described in Materials and Methods.

† Rats were fasted for 24 hr.

‡ Hearts obtained from fed rats were perfused 1 hr with cobaltous chloride at the indicated concentration.

§ Denotes significant decrease from fed control ($P < 0.05$).

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