# STIMULATING EFFECT OF HAEMIN ON THE SYNTHESIS OF CYTOCHROME *C* BY LIVER SLICES

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## 1. Introduction

Several laboratories have reported that haemin or its precursors stimulate haemoglobin synthesis in intact reticulocytes or in cell-free systems, by acting at the translational level (see, among others, [1–7]). A similar regulatory effect, although at the transcriptional level, has been proposed for the case of yeast cytochrome *c* [8, 9]. The prosthetic group of this latter protein differs from protohaem only in the existence of two thioether bridges between the reduced vinyl chains of the tetrapyrrol ring and two cysteine residues of the polypeptide chain. In the present communication we report results showing that haemin enhances the synthesis *in vitro* of the apoprotein moiety of cytochrome *c* in liver slives from five-day-old rats, and that this effect is inhibited by actinomycin D.

## 2. Material and methods

L-U-$^{14}$C-lysine (320 mCi/mM) and $^{59}$Fe-citrate (600 mCi/mM) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Haemin chloride (bovine) was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.; actinomycin D from Mann Research Laboratories, New York; and allyl-isopropylacetamide (AIA) was a gift from Hoffman La Roche Inc. Co., Basle. Five-day-old rats (Sprague Dawley, 12.5 ± 1.5 g body wt) were used throughout, in order to ensure a high rate of mitochondrial formation in the liver [10].

Slices were incubated and homogenized for cytochrome *c* and total protein preparation as detailed in fig. 1. Protein was precipitated from 0.5 ml aliquots of the homogenates by the addition of trichloroacetic acid to a final concentration of 10% w/v, and processed as previously described [11], in the presence of unlabelled lysine and ferric citrate. The protein was finally dissolved in formic acid and estimated by plating 0.2 ml aliquots on pre-weighed aluminium disks, drying under an infrared lamp and weighing. The concentration was then adjusted to 10 mg/ml and samples of 0.2 ml were mixed with 10 ml of scintillation fluid "tT21" [12]. The determinations of $^{14}$C and $^{59}$Fe were performed by double channel counting and values for $^{59}$Fe were corrected for decay back to the incubation date.

Cytochrome *c* was extracted from the remainder of the homogenates of the liver slices by the procedure for unfractionated liver previously described [13], modified as follows: (a) the adsorption on Amberlite resin at pH 7.0 was carried out on an 8 ml column and the 0.15 M NaCl extract (six-fold diluted) was adsorbed on the same Amberlite column immediately after the passage of the water extract in order to process a single cytochrome *c* fraction for each sample; (b) the adsorption on Amberlite resin at pH 9.0 was omitted and the absence of contaminating pigments checked by recording the oxidized and reduced spectra; (c) 5 mg of ferric citrate and 1 mg of chromatographically pure rat-liver cytochrome *c* (as a carrier) were always added, together with the unlabelled L-lysine immediately before the gel filtration step; (d) the precipitate obtained with $(NH_4)_2SO_4$ and trichloroacetic acid, was dissolved

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in 3 ml of 0.1 M ammonium acetate and the spectrum was recorded before and after adding sodium dithionite. Trichloroacetic acid was added to a 20% (w/v) final concentration; the cytochrome *c* was collected by centrifugation at 10,000 *g* for 20 min, and was dissolved in 0.4 ml of formic acid. The radioactivity was determined as in the case of total protein, using 15 ml of scintillation fluid.

### 3. Results and discussion

The incorporation by the liver slices of $^{14}$C-lysine into total protein and cytochrome *c* is linear over the first two hr of incubation (fig. 1). The incorporation of $^{59}$Fe into cytochrome *c* showed a significant lag up to 60 min, and thereafter the rate appeared similar to that of $^{14}$C incorporation. This delay was not observed in the case of total protein. The lag shown for cytochrome *c* could be due to a pool of unlabelled haem, and/or to the subcellular compartmentation of both substances, whereas in the incorporation into total protein the mechanism involved is mainly the chelation of $^{59}$Fe by apoferritin.

The results of the experiments on the effect of haemin are shown in table 1. A 30 min pre-incubation of the liver slices with 0.23 mM haemin remarkably stimulated the incorporation of $^{14}$C-lysine into cytochrome *c* during a 30 min period of incubation with the radioactive precursors (Exp. 1). This effect was abolished when the addition of haemin was preceded by another 30 min pre-incubation with 8 μM actinomycin D. The antibiotic did not significantly affect the $^{14}$C specific radioactivity of cytochrome *c* in the absence of haemin.

The stimulation by haemin was also observed at 60 min (Exp. 2) and 120 min (Exp. 3) of incubation with radioactivity, and the effect of actinomycin D at this last period was quite similar to that observed at 30 min. Both the increase in incorporation of $^{14}$C-lysine induced by haemin and the prevention of this stimulation by the antibiotic appeared to be restricted to cytochrome *c*, since the $^{14}$C specific radioactivity of total protein was not significantly changed. On the other hand, the insertion of $^{59}$Fe into cytochrome *c* was reduced by the addition of haemin, probably due to the dilution of the $^{59}$Fe-haem.

AIA induces the synthesis of σ-aminolevulinic synthetase, the enzyme that controls the rate of synthesis

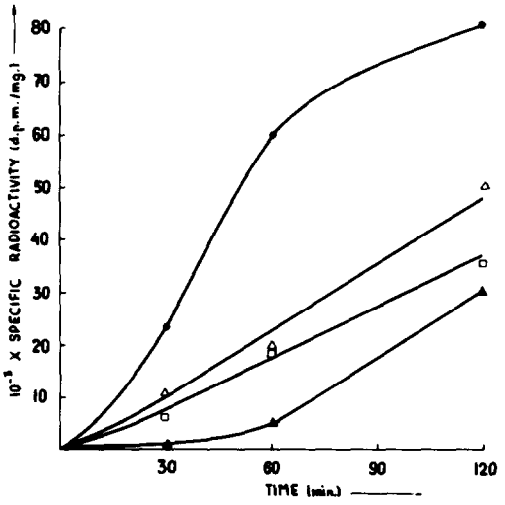


Fig. 1. Incorporation of $^{14}$C-lysine and $^{59}$Fe into cytochrome *c* and total protein by liver slices from five-day-old rats. Groups of liver slices (3.4–3.8 g) from 10–12 rats were pre-incubated with 30 ml of the medium of Peters and Anfinsen [14] under 95% $O_2$–5% $CO_2$ in 250 ml conical flasks, at 37°, for 30 min. The medium was then decanted and replaced by an equal volume of the same medium supplemented with a mixture of all 20 amino acids (0.25 mM each) with the exception of lysine. After incubation for 30 min at 37°, 2.5 ml of 0.9% NaCl containing 30 μCi of $^{14}$C-lysine and 200 μCi of $^{59}$Fe-citrate was added to each flask. After various time intervals the reaction was stopped by the addition of unlabelled precursors (5 mg each of lysine and Fe-citrate in 1 ml of 0.9% NaCl) and cooling in ice. The slices were separated from the medium by centrifugation at 5,000 *g* for 10 min and washed twice further by resuspension in 30 ml of 0.25 M sucrose containing the unlabelled precursors. Each group of slices was homogenized in 20 ml of distilled water and the total protein and cytochrome *c* prepared for radioactivity determinations as described in the text. Values are averages of two separate experiments.
□, Total protein ($^{14}$C); ●, total protein ($^{59}$Fe); △, cytochrome *c* ($^{14}$C); ▲, cytochrome *c* ($^{59}$Fe).

of haem, and hence of haemoglobin [15, 16]. However, the drug promoted only a slight stimulation of the incorporation of $^{14}$C-lysine into cytochrome *c* under conditions where the synthesis of haem was apparently enhanced, as shown by the higher specific radioactivity of $^{59}$Fe in cytochrome *c* (Expts. 2 and 3).

Our results with haemin suggest that the prosthetic group of cytochrome *c* controls the rate of synthesis of the apoprotein moiety. The experiments with actinomycin D seem to indicate that the stimulation by haemin was mediated through the formation of new

Table 1
Effects of haemin, allyl-isopropylacetamide and actinomycin D on the synthesis of cytochrome *c* and total protein by liver slices.

| Exp. No | Samples | Incubation time with $^{14}C$ and $^{59}Fe$ (min) | $10^{-3}$ X Specific radioactivity (dpm/mg) Cytochrome *c* $^{14}C$ | Cytochrome *c* $^{59}Fe$ | Total protein $^{14}C$ |
|---|---|---|---|---|---|
| 1 | Control | 30 | 10.4 | < 0.5 | – |
| 1 | Haemin | 30 | 23.4 | < 0.5 | – |
| 1 | Actinomycin D | 30 | 12.0 | < 0.5 | 13.8 |
| 1 | Actinomycin D + haemin | 30 | 7.0 | < 0.5 | 11.9 |
| 2 | Control | 60 | 18.2 | 5.1 | 17.7 |
| 2 | Haemin | 60 | 35.8 | 2.0 | 15.5 |
| 2 | AIA | 60 | 25.0 | 9.1 | 19.9 |
| 3 | Control | 120 | 51.2 | 31.1 | 35.1 |
| 3 | Haemin | 120 | 87.4 | 28.3 | 38.3 |
| 3 | AIA | 120 | 59.3 | 52.0 | 39.0 |
| 3 | Actinomycin D | 120 | 47.3 | 18.8 | 35.0 |
| 3 | Actinomycin D + haemin | 120 | 54.9 | 24.4 | 31.1 |

The experiments were performed as described in fig. 1, except that before the addition of radioactive precursors, the 30 min incubation of the slices with fresh medium was carried out with or without haemin (0.23 mM) or AIA (2 mM). In the experiments with actinomycin D (8 μM) these treatments were preceded by a 30 min incubation in the presence of the antibiotic.

mRNA, since no side effects on total protein synthesis [17] were observed. This would imply a control at the level of transcription, as opposed to the case of haemoglobin synthesis which appears to be regulated at the translation mechanism [1, 2, 5, 6]. Further work is in progress to define more precisely the site of haemin action on the synthesis of cytochrome *c*.

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