EUFLAVIN AND ETHIDIUM BROMIDE; INHIBITORS OF MITOCHONDRIOGENESIS IN REGENERATING RAT LIVER

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1. Introduction

We have described earlier that chloramphenicol and oxytetracycline block the formation of the mitochondrial enzyme cytochrome *c* oxidase in regenerating rat liver almost completely [1–3]. These antibiotics also inhibit rat-liver mitochondrial protein synthesis *in vitro* [4] and cytochrome *c* oxidase formation in cultured rat-heart cells [2, 5]. Apparently the mitochondrial translation system is involved in the formation of functionally active cytochrome *c* oxidase [2, 3]. Hence, we were interested to know whether the mitochondrial genome is also necessary for the formation of this enzyme. The best way to investigate this point seemed to us the use of the trypanocidal dyes euflavin and ethidium bromide, since these are known to inhibit mitochondrial RNA-synthesis [6–8]. This inhibition is caused by the intercalation of the dyes into double-stranded DNA [9]. Furthermore, low concentrations of the dyes completely inhibit mitochondrial protein synthesis *in vitro* [10, 11]. This paper describes the effects of low concentrations of euflavin and ethidium bromide on mitochondrial cytochrome *c* oxidase formation in regenerating rat-liver; the results of these experiments show that the formation of this respiratory enzyme is inhibited by the intercalating dyes also *in vivo*.

2. Materials and methods

Male Wistar rats, weighing about 200 g, were used throughout. The method of partial hepatectomy, the assays of cytochrome *c* oxidase (EC 1.9.3.1) activity and protein, the preparation of rat liver homogenates and mitochondria and the spectrophotometric measurement of cytochrome concentrations in mitochondria were the same as described previously [3].

3. Results

The results with homogenates from regenerating livers are summarized in fig. 1. Regenerating livers were excised 46 hours after partial hepatectomy. The drugs were injected only once, either intravenously or intraperitoneally at the time of the operation. As can be seen from fig. 1a, the protein content of livers from treated animals did not differ significantly from control values, showing that regeneration in total was not influenced by the dyes. The total cytochrome *c* oxidase activity, however, was markedly depressed by the treatment with euflavin or ethidium bromide (fig. 1b). Euflavin was injected intravenously or intraperitoneally at a dose of 5 or 10 μg per body weight and gave a fairly strong inhibition of cytochrome *c* oxidase formation for both concentrations and in both ways of application. The results for euflavin have therefore been grouped together into one column. For ethidium bromide (10 μg per g body weight), there was a difference in inhibition depending on the way of administration: intraperitoneal injection consistently gave a stronger inhibition of cytochrome *c* oxidase formation than intravenous application did. Moreover, within the group of intravenously injected animals a much higher variability of the degree of inhibition

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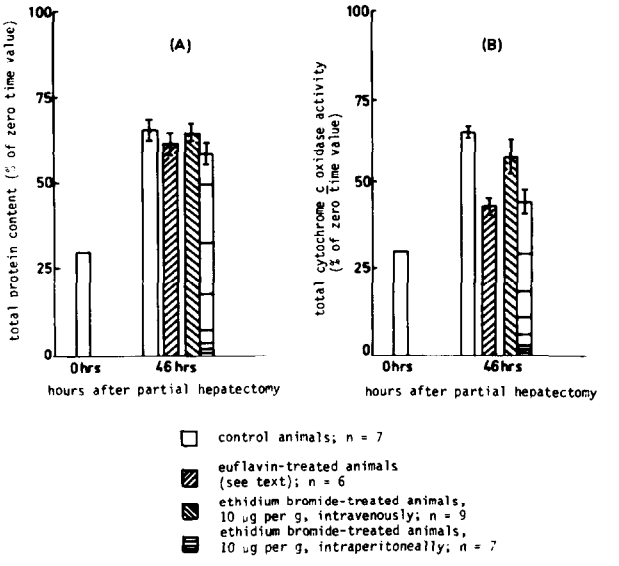

Fig. 1. (a) Total protein content of regenerating rat-liver. 100% is about 900 mg. The values for the part of the liver excised during partial hepatectomy (zero time) are taken as 70%. Standard errors are indicated by vertical bars. Each column is cross-hatched as indicated in the figure.

(b) Total cytochrome *c* oxidase activity of regenerating rat-liver. 100% activity is about 25,000 min^{-1}. Cross-hatching is the same as fig. 1a.

existed than was the case for euflavin-treated or intraperitoneally injected ethidium-treated animals.

We also have determined the ratios of cytochrome aa_3 to cytochromes $c + c_1$ in mitochondria prepared from regenerating liver. Fig. 2 gives the ratios for mitochondria from control and treated rats. It is clear that in the mitochondria of euflavin - or ethidium bromide-treated rats the concentration of cytochrome aa_3 (the hemoprotein identical to cytochrome *c* oxidase) is lowered as compared to the extramitochondrially synthesized [12] cytochrome *c*. For chloramphenicol - or oxytetracycline-treated rats the concentration of cytochromes $c + c_1$ per mg protein in mitochondria from regenerating liver was about 25% higher than for control animals [3]. We have ascribed this to a decrease in the synthesis of some other mitochondrial proteins in the antibiotic-treated rats. In the experiments presented here, this difference is not that pronounced. For the dye-treated animals the concentration of cytochromes $c + c_1$ was about 0.42 nmoles per mg mitochondrial protein, whereas this value was about 0.38 for mitochondria from normal livers and from regenerating livers of untreated rats.

Summarizing, the intercalating dyes, although injected only once and in low concentrations, exert a specific inhibitory effect on the formation of the mitochondrial enzyme cytochrome *c* oxidase also in the living animal.

4. Discussion

The results reported in this paper are completely in line with the inhibitions observed on mitochondrial protein synthesis *in vitro* [4, 11] and on cytochrome *c* oxidase formation in cultured heart cells [11]. To get inhibition of cytochrome *c* oxidase in regenerating liver or heart cells by chloramphenicol or oxytetracycline, it was necessary to keep the serum level or the concentration in the culture medium continuously at or above 5–10 µg/ml, being the bacteriostatic level [1, 3, 5]. Moreover, when the antibiotics were with-

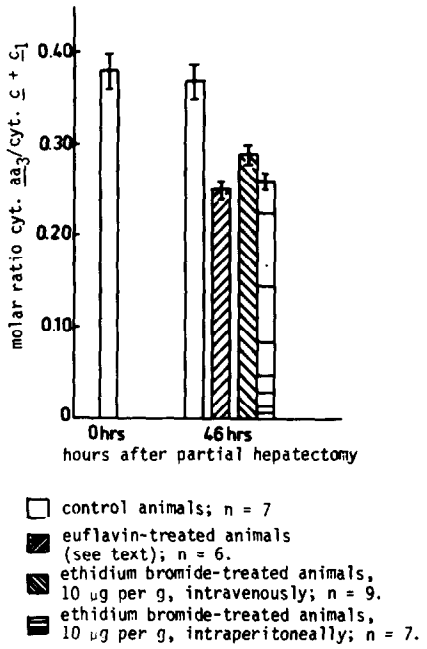

Fig. 2. Molar ratios of cytochrome *aa3* to cytochromes *c* + *c1* in mitochondria from regenerating rat-liver. For the calculation of the cytochrome concentrations, the mmolar extinction coefficient 24.0 for cytochrome *aa3* given by Van Gelder [16] and 17.8 for cytochromes *c* + *c1* (2 : 1) (J.Berden, Personal Communication) have been used. Cross-hatching is the same as for fig. 1.

drawn from the animals or the culture medium, immediate reversal of cytochrome *c* oxidase formation to control levels occurred. On the contrary, as is described above, only one injection of euflavin or ethidium bromide is already sufficient to achieve inhibition of cytochrome *c* oxidase formation over a period of 2 days of regeneration. This also holds for the cultured heart cells: 6 days after changing from dye-containing to normal medium cytochrome *c* oxidase formation is still completely blocked [11]. In our opinion the persistency of the inhibition over a long period points to an irreversible damage of the mitochondrial biosynthetic machinery by euflavin and ethidium bromide.

The variability in degree of inhibition by ethidium bromide observed after intravenous injection has most likely to be ascribed to the lipophilic character of the dye: if all the dye molecules have been titrated away by tissue and serum lipids before they can reach the liver, no effect on the liver mitochondria can be expected. Furthermore, we have to presume that a large fraction of the mitochondrial DNA population must be altered by the drug before the formation of cytochrome *c* oxidase will be inhibited severely. Unfortunately, we have not been able so far to correlate the decrease in cytochrome *c* oxidase formation to the actual concentrations of the dyes in blood and liver. Fluorimetric and spectrophotometric measurements available to estimate euflavin and ethidium bromide concentrations are not sensitive enough for this purpose. For the same reason we have not yet been able to look into the fate of the dyes with respect to biotransformation and excretion.

To exclude the possibility that the dyes act directly on the level of translation, we have tested euflavin and ethidium bromide in control systems, *viz.* in rat-liver microsomes and in an *E. coli* S-30 fraction [13]. In neither of these systems any inhibition on protein synthesis was found for the concentrations of euflavin and ethidium bromide that gave 90–100% inhibition of mitochondrial protein synthesis [11]. Other investigators have found an inhibition of ethidium bromide on aminoacylation of tRNA [14] and a binding to tRNA [15]. At the concentration at which almost complete inhibition of mitochondrial protein synthesis is found, however, ethidium bromide gave only a very weak effect in their experiments. Therefore, and also in view of the apparent irreversibility of the *in vivo* inhibition, a direct effect of the dyes on mitochondrial translation seems very improbable.

We can conclude from these experiments that the formation of cytochrome *c* oxidase *in vivo* is not only depending on the mitochondrial translation system but also on the mitochondrial genome. Though conclusions about the localization of the genetic message for the apoenzyme of cytochrome *c* oxidase can not be drawn from this type of results, as we have stressed earlier [2, 3].

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