THE EFFECTS OF INHIBITORS OF PROTEIN SYNTHESIS ON THE SYNTHESIS OF HEME IN RABBIT RETICULOCYTE

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The syntheses of heme and of globin have been shown to proceed in parallel in reticulocytes of the rabbit (Kruh and Borsook, 1956) and the dog (Nizet, 1957) and in erythroid cells of rabbit bone marrow (Morell et al. 1958). The coordination of these syntheses appears to result from inhibition by heme of its own synthesis (Karibian and London, 1965) and stimulation by heme of the synthesis of globin (Bruns and London, 1965, Grayzel et al, 1966). In a further study of the relationships between these two synthetic processes we have examined the effects of inhibiting protein synthesis on the formation of heme.

Methods

Reticulocytosis was produced in rabbits made anemic by the injection of acetylphenylhydrazine, 10mg per kg for five days. The rabbits were bled on the seventh day. The blood was centrifuged at 1000 x g for 10 minutes, the plasma removed and the cells resuspended in one volume of the same plasma. Each incubation flask contained 4 ml of the cell suspension, 4 ml of a salt solution (0.13M NaCl, 0.0052M KCl, 0.0075 M $MgCl_2$) containing glucose (6mg/ml), NaHCO3 (0.66 mg/ml)and a mixture of amino acids (Lingrel and Borsook, 1963). The mixture was adjusted to pH 7.7.

For studies of heme synthesis 5 microcuries of glycine-2-C^{14} (20 mc/mM) or ALA-4-C^{14} (20mc/mM) from New England Nuclear Corp.were added to the incubation medium; for the determination of protein synthesis, 2 microcuries of L-leucine-U-C^{14} (250 mc/mM New England Nuclear Corp.) were added to the incubation

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medium. Cycloheximide was dissolved in the salt solution and added in the concentrations indicated. The incubation was carried out in a 37°C water bath in stoppered flasks.

At the end of the incubation the cells were washed three times in cold isotonic NaCl solution and lysed with one volume of distilled water. Non-radioactive red cell hemolysate (10 ml.) was added to each flask to provide carrier heme. Hemin was crystallized from the total hemolysate by the glacial acetic acid method of Fischer (1941) and recrystallized from pyridine-chloroform. The radioactivity of weighed samples was measured at infinite thickness in a Nuclear Chicago gas flow counter. All experiments were performed in duplicate with agreement within +/-5 per cent. The specific activity of the hemoglobin from cells incubated with leucine-C^{14} was determined by measuring the concentration of hemoglobin spectrophotometrically and precipitating 1 mg of protein on millipore filters to measure radioactivity as previously described (Grayzel et al, 1966). More than 95 per cent of the protein synthesized by reticulocytes consists of globin. Adenosine triphosphate (ATP) content was measured by the method of Gross et al. (1963).

Results

When reticulocytes are incubated under conditions in which the synthesis of protein is inhibited almost completely, the utilization of glycine for the synthesis of heme is markedly diminished whereas the utilization of delta amino-levulinic acid (ALA) is only slightly reduced. Table 1 shows the effects of cycloheximide and puromycin on the utilization of glycine and of ALA for the synthesis of heme during a 4 hour incubation. These effects are similar to the effects of added hemin (Karibian and London, 1965).

There is a progressive inhibition of the utilization of glycine for the synthesis of heme as the synthesis of protein or globin declines (Fig. 1). When protein synthesis ceases, the utilization of glycine for the synthesis of heme is reduced to half the control value.

Table 1

Inhibition of Heme and Protein Synthesis by Cycloheximide and Puromycin

Exp.	Inhibitor	Protein L-leucine-U-C^{14}		Heme Glycine-2-C^{14}		Heme ALA-4-C^{14}	
		cpm/mg	% of control	cpm/mg	% of control	cpm/mg	% of control
1	None	221		4690		6926	
	Cycloheximide (2×10^{-5}M)	7	97	1794	62	5929	14
2	None	154		2530		1996	
	Cycloheximide (2×10^{-5}M)	8	95	1260	50	1916	4
3	None	245		1133		5429	
	Puromycin (2×10^{-3}M)	1	100	625	45	4825	11
(a)	Hemin (1×10^{-4}M)				48.6 ±2.0 (S.E.)		12.2 ± 4.8 (S.E.)

(a) Data from Karibian and London (1965)

EFFECT OF CYCLOHEXIMIDE ON THE SYNTHESIS OF HEME AND GLOBIN

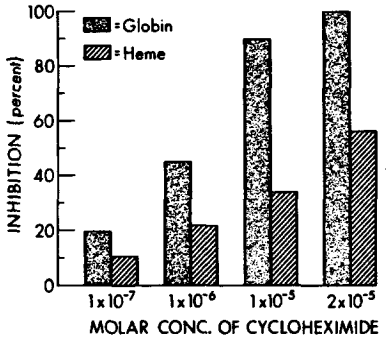

Fig. 1

Synthesis of protein virtually ceases within a few minutes after the addition of cycloheximide (2×10^{-5} M) to reticulocytes. Within 15 minutes after the addition of cycloheximide, perceptible inhibition of the utilization of glycine for heme synthesis occurs and continues throughout the 2-hour period of incubation (Fig. 2.).

UTILIZATION OF GLYCINE-2-C^{14} FOR HEME SYNTHESIS

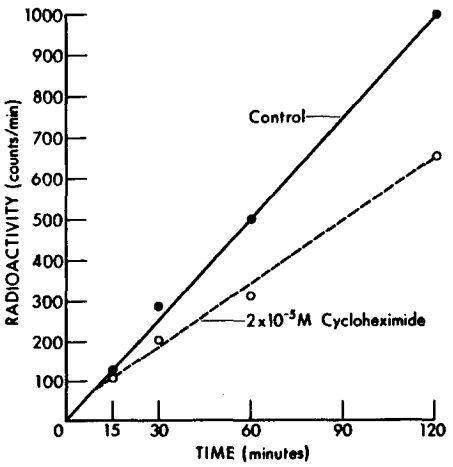

Fig. 2

Measurement of the content of ATP in these cells in the course of a 4-hour period of incubation showed a slight decline of equal degree in the control and cycloheximide-treated cells (Table 2).

Table 2

Effect of Cycloheximide (2×10^{-5}M) on the ATP Content of Rabbit Reticulocytes

Incubation Time	ATP content (μM/g hemoglobin)	
min.	control	cycloheximide 2×10^{-5}M
0	8.25	8.25
30		7.39
240	6.63	6.60

Discussion

The inhibition of heme synthesis by cycloheximide or puromycin is very similar to that observed when reticulocytes are incubated with added hemin. The utilization of glycine for heme synthesis is markedly reduced, whereas the utilization of ALA is much less inhibited. The inhibitory effects on heme synthesis are exerted on one or more steps prior to the formation of ALA.

Cycloheximide and puromycin might influence the formation of ALA in several ways. They might exert a direct inhibitory effect on enzymic function, but there is no evidence at present in support of this possibility. They might affect the activity of mitochondria which are required for the formation of ALA; cycloheximide, however, has been shown not to affect mitochondrial metabolism adversely (Lardy et al. 1958, Sisler and Siegel, a, 1967). They might inhibit the synthesis of one or more enzymes involved in the formation of ALA. The rapid effect of cycloheximide would require that such enzymes have a very rapid turnover. Since a reticulocyte hemolysate can continue to synthesize heme from glycine for several hours (Karibian and London, 1965) in the face of nearly complete cessation of protein synthesis (Lamfrom and Knopf, 1964), it is unlikely that very rapid turnover of these enzymes occurs in reticulocytes.

The most likely interpretation of these findings, we believe, is that the inhibition of protein synthesis results in a deficiency of globin which constitutes more than 90 per cent of the protein synthesized in the reticulocyte. The lack of globin as an acceptor for heme would cause newly synthesized heme

to accumulate within the cell and to reach a concentration which is inhibitory to ALA formation and heme synthesis. The rapid onset of the effect suggests that an inhibitory concentration of heme may be reached very quickly at a critical site, such as within the mitochondria. This interpretation favors the view that the synthesis of globin plays a role in regulating the synthesis of heme (London, 1965).

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