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Cycloheximide as a Probe of Fibrinogen Synthesis

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Fibrinogen synthesis in the intact rat was perturbed by treatment with cycloheximide. Specific radioactivities of fibrinogen in plasma and liver both decreased at 2h after treatment and increased over 2-fold by 18h. Labelled-antibody–polyribosome binding experiments showed that more polyribosomes were engaged in fibrinogen synthesis at 18h after treatment. Radioactivity of plasma fibrinogen chains from untreated control rats showed a constant ratio of $A\alpha$–$B\beta/\gamma = 1.03$. At 2h after cycloheximide treatment the $A\alpha$- and $B\beta$-chains showed the greatest decrease in labelling ($A\alpha$–$B\beta/\gamma = 0.66$) and at 18h all chains were much more labelled (the $A\alpha$–$B\beta/\gamma$ ratio changed to 1.39). The observed imbalance in fibrinogen-chain synthesis suggests that cycloheximide has a selective effect on gene expression.

The rate of synthesis and plasma concentrations of fibrinogen change in response to many conditions causing tissue damage and inflammation (Koj, 1970), such as treatment with turpentine (Weimer & Humelbaugh, 1967; Kwan & Fuller, 1977) or pyrogen (Bocci *et al.*, 1976). We have reported that a single injection of a non-lethal dose of cycloheximide into rats alters the rate of plasma fibrinogen synthesis. Synthesis was drastically inhibited at 2h, but at 12 and 18h after the antibiotic administration there was a 3-fold stimulation (Ch'ih *et al.*, 1977*a*). This biphasic phenomenon led us to investigate the possible molecular mechanism of fibrinogen biosynthesis in the intact rat using cycloheximide as a perturbing agent. A preliminary report on part of this work has been presented (Procyk *et al.*, 1978).

Experimental

The experiments were performed on male Wistar rats (210±10g). Maintenance of the animals, treatment with cycloheximide, collection of plasma and removal of livers were carried out as described by Ch'ih *et al.* (1977*a*). Plasma fibrinogen was purified by the method of Bouma & Fuller (1975) and analysed by acid/urea/polyacrylamide-gel electrophoresis (Brummel & Montgomery, 1970). Fibrinogen chains were reduced, alkylated and separated by CM-cellulose chromatography (McDonagh *et al.*, 1972). Anti-(rat fibrinogen) antibodies were purified from rabbit antisera by affinity chromatography on columns of fibrinogen–Sepharose (Bouma & Fuller, 1975). Iodination was carried out with Bolton–Hunter Reagent (Amersham, Arlington Heights, IL, U.S.A.). Isolation of liver cytoplasmic ribonucleoprotein complexes and polyribosome separation were performed as described by Ch'ih *et al.* (1977*b*). Supernatants of the fractions from which ribonucleoprotein complexes were isolated were titrated with anti-fibrinogen, and, at the equivalence point, precipitated antibody–fibrinogen complexes were collected by centrifugation (5000*g* for 10min). Radioactivity was determined as described by Devlin & Ch'ih (1974). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and Discussion

In the first phase of this investigation, experiments were performed to assess the effect of cycloheximide on fibrinogen synthesis. The goal of these initial studies was not to measure the rate of synthesis, as this has been determined previously (Ch'ih *et al.*, 1977*a*). Rather, the goal was to develop a consistent picture of liver fibrinogen synthesis when this process was perturbed by cycloheximide.

Table 1 presents specific radioactivities of fibrinogen in plasma and liver. Both sets of data show decreased fibrinogen synthesis at 2h and stimulation at 18h after cycloheximide treatment. The purified plasma fibrinogen values were lower than those obtained earlier by thrombin precipitation (Ch'ih *et al.*, 1977*a*); however, this is presumably a reflection of fibrinogen accumulation in the plasma. The 2.3-fold stimulation of fibrinogen labelling observed here at 18h after cycloheximide administration, when corrected by 18% for accumulation (Ch'ih *et al.*, 1976), rises to 2.8-fold, a value in agreement with the earlier data. Interestingly, the relative ratio of fibrinogen specific radioactivity in plasma to that in

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Table 1. *Effect of cycloheximide on fibrinogen biosynthesis*

Animals were treated with [^{3}H]leucine for 1 h before being killed. Plasma and liver fibrinogen were isolated as described in the Experimental section. The data are representative of typical experiments; each experiment consisted of pooled samples from six to eight rats.

Experimental conditions	Radioactivity: Purified plasma fibrinogen (d.p.m./mg of protein)	(Percentage of control)	Liver fibrinogen immunoprecipitate (d.p.m./A_{260} of post-polyribosomal supernatant)	(Percentage of control)
Control	6695	100	590	100
Cycloheximide-treated				
2h	1071	16	162	27
18h	15705	235	1344	228

liver falls at 2h, but returns to the control value by 18h after cycloheximide treatment. This may indicate changes in the rate of secretion of this plasma protein; however, at present, it is rather difficult to give an unambiguous explanation for the decreased ratio at 2h.

The binding of ^{125}I-labelled anti-fibrinogen to liver polyribosomes was used to identify the region of a polyribosomal profile that was engaged in fibrinogen synthesis (Bouma *et al.*, 1975). This type of experiment was conducted to determine the effect of cycloheximide on antibody–polyribosomal binding. Labelled antibody was incubated with polyribosomes from untreated and cycloheximide-treated animals (Fig. 1). In all cases a significant peak of radioactivity appeared in the heavy-polyribosomal region. The material in this peak was sensitive to ribonuclease and unaffected by subsequent additions of fibrinogen and anti-fibrinogen to the reaction mixture (results not shown). Greater amounts of antibody were bound to polyribosomes from animals 18h after treatment with cycloheximide than to control polyribosomes (about 133% of control). At 2h after treatment, less labelled antibody was bound (66% of control). This implies that during the period of stimulated protein synthesis there were more polyribosomes engaged in fibrinogen synthesis, and therefore the rate of label incorporation increased. The opposite would be true for the circumstances after 2h.

Although substantial changes in the rate of fibrinogen synthesis after cycloheximide treatment have been demonstrated, some technical problems complicated our attempts to assess qualitative differences in the antibody–polyribosomal binding for the treated conditions. Some antibody bound to the light-polyribosomal region in samples from 18h-treated animals; however, the significance of this binding is not yet understood. Since fibrinogen is composed of three different polypeptide chains (Blomback & Yamashina, 1958), synthesis may involve different polyribosomal regions. To investigate this hypothesis we speculated that individual polypeptide chains of fibrinogen may be synthesized at different rates after perturbation of normal synthesis by cycloheximide. Therefore experiments that measured

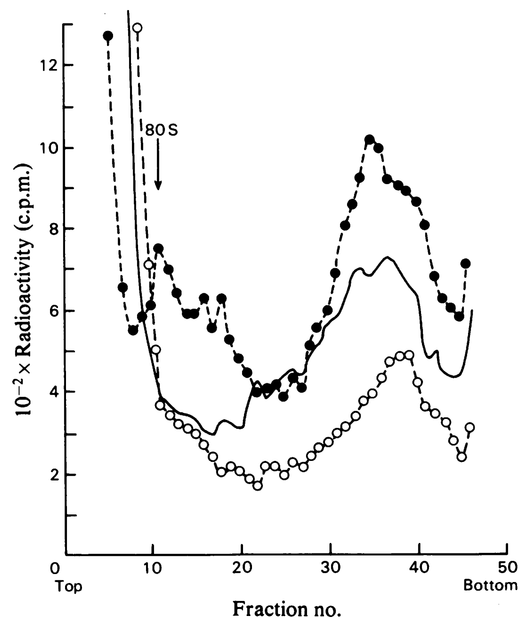

Fig. 1. *Binding of ^{125}I-labelled anti-fibrinogen to liver polyribosomes*

Total incubation volume was 0.6ml. The mixture (containing 17.5 μg of antibody and 10 A_{260} units of polyribosomes) was layered over a linear sucrose gradient (0.5–1.5 M) and centrifuged for 195 min at 96300 g in a Spinco SW 27 rotor at 4°C. Gradients were withdrawn from the top and monitored continuously at 260 nm. Absorbance was recorded and fractions (50 drops) were collected for determination of radioactivity. ——, Control; ○, treated with cycloheximide for 2h; ●, treated with cycloheximide for 18h.

the rate of [^{3}H]leucine incorporation into the fibrinogen chains were conducted.

Rates of [^{3}H]leucine incorporation into fibrinogen chains, Aα–Bβ–γ, were determined in samples from selected regions on the CM-cellulose-elution profile of reduced alkylated rat plasma fibrinogen (Fig. 2). The presence of γ-chain in the first peak and Aα–Bβ-chains in the second peak was demonstrated by polyacrylamide-gel electrophoresis (results not shown). The ratio of specific radioactivities of the peak fractions from the Aα- and Bβ-chain region to the γ-chain region was close to unity for fibrinogen purified from untreated rats (Table 2). With two different [^{3}H]leucine dosages for control animals, the specific radioactivities increased proportionally in both fractions with the ratio remaining constant, indicating that leucine incorporation into the fibrinogen chains is dependent on the amount of label administered and the ratio is independent of the amount. The ratios of 1.02 and 1.04 obtained for the untreated animals approach the calculated ratio determined by dividing each peak area in Fig. 2 by the leucine composition of the fibrinogen chains in that peak (Bouma & Fuller, 1975; Van Ruijven-Vermeer & Nieuwenhuizen, 1978). The ratio of the calculated value of Aα- and Bβ-chain to γ-chain leucine content is about 0.92; our experimentally determined value was 1.02–1.04.

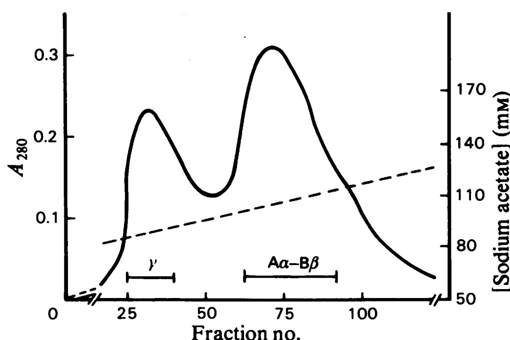

Fig. 2. *CM-cellulose chromatography of reduced alkylated rat plasma fibrinogen*

Relative peak areas (from planometric analysis of the elution profiles of five chromatographs) are γ-chain, 34±0.5; Aα–Bβ-chains, 66±0.9. ——, A_{280}; ----, concentration of sodium acetate (mM).

As shown in Table 2, fibrinogen-chain synthesis after cycloheximide administration was first inhibited and then stimulated, a response similar to that exhibited by the intact molecule (Table 1). Interestingly, the ratio of the specific radioactivities of the chains at 2h changed from 1 to 0.66, reflecting a greater decrease in Aα–Bβ-chain labelling. In contrast, this ratio rose to 1.39 at 18h after cycloheximide treatment, suggesting an increased synthesis of Aα–Bβ-chains. These findings indicate that an imbalance of fibrinogen-chain synthesis may occur during the changing rate of protein synthesis after cycloheximide perturbation of the system.

Since a uniform rate of chain labelling was not observed when protein synthesis was perturbed by cycloheximide, it seems probable that the asymmetric incorporation of [^{3}H]leucine into fibrinogen chains resulted from differential activation of the fibrinogen genes. Similar findings were reported by Alving *et al.* (1977) in experiments that measured [^{75}Se]-selenomethionine incorporation into fibrinogen chains of turpentine-treated rabbits. It is possible that separate mRNA molecules may exist for each fibrinogen polypeptide chain, with cycloheximide having a selective effect on gene expression. The existence of this effect provides the means to investigate further the molecular mechanism of fibrinogen biosynthesis.

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Table 2. *Incorporation of [^{3}H]leucine into polypeptide chains of fibrinogen after cycloheximide treatment*

Animals were treated with [^{3}H]leucine for 1h before being killed. Fibrinogen chains were separated by CM-cellulose chromatography. Six animals were used in each experiment. The data are representative of typical experiments.

Time after cycloheximide treatment (h)	[^{3}H]Leucine dose (μCi/100g body wt.)	Radioactivity in the peak fractions (d.p.m./A_{280} unit)		Ratio Aα–Bβ/γ
		γ-chain	Aα- and Bβ-chains	
0	40	1965	2047	1.04
2	40	376	249	0.66
18	40	4018	5594	1.39
0	80	3978	4070	1.02

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