THE EFFECTS OF AFLATOXIN B_1 AND STEROID HORMONES ON POLYSOME BINDING TO MICROSOMAL MEMBRANES AS MEASURED BY THE ACTIVITY OF AN ENZYME CATALYSING DISULPHIDE INTERCHANGE

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An enzyme catalysing the rearrangement of protein disulphide bonds has been found firmly attached to the endoplasmic reticulum of all tissues tested [1–4]. The biological function of the enzyme is uncertain although it is probably involved in terminal stages of the biosynthesis of secreted and lysosomal proteins, many of which possess disulphide bonds. The apparent interchange activity of microsomal membranes, measured by their ability to catalyse the reformation of native, active enzyme from "randomly-oxidised" ribonuclease [1], is a function of the number of ribosomes bound [4] and there appears to be an equivalence between the amount of interchange enzyme and the number of polysome-binding sites. Fig. 1 shows the relationship between the apparent interchange activity (per mg microsomal protein) and the RNA: protein ratio for several sub-fractions of three different tissues, all taken from the same male, albino rats (150–220 g body weight). The plots are linear and parallel, and the parameters obtained from these plots are given in table 1. It would seem that polysome-binding sites are uniquely associated with loci of interchange enzyme activity and it is suggested that this enzyme catalyses the formation of the correct conformation of proteins "exported" from the cell via the endoplasmic tubules.

The effects of aflatoxin B_1 on the relationship between the enzyme and polysome binding were investigated *in vitro*, since in common with most carcinogens it appears to induce "degranulation" of the endoplasmic reticulum *in vivo* [5]. It is also an inhibitor of protein synthesis in liver slices [6] and causes a breakdown of polysomes in HeLa cells [7].

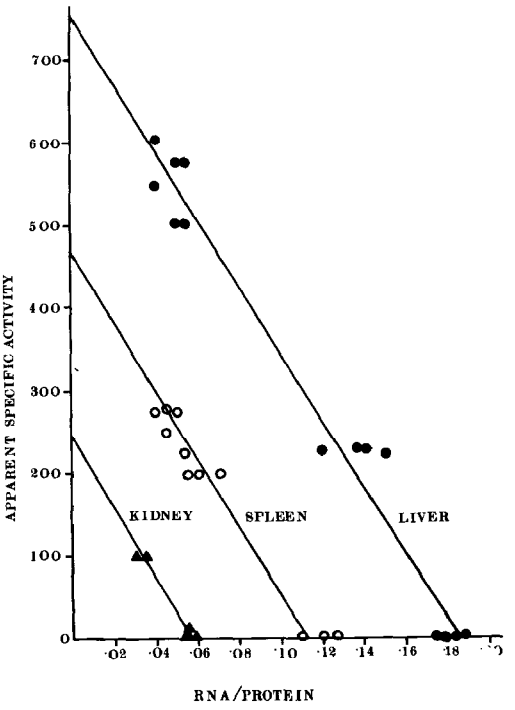

Fig. 1. The relationship between the apparent disulphide interchange activity and the RNA:protein ratio for various microsomal sub-fractions of three different tissues: –●– liver; –○– spleen; –▲– kidney. Protein was estimated by the method of Lowry et al. [9] and RNA by a modification of the method of Monro and Fleck [10], using the relationship $\epsilon^{1\%}_{1\,cm} = 300$ for hydrolysed RNA. Microsomes were prepared and sub-fractionated by a modification of the method of Bloemendal et al. [11].

Table 1

Tissue	Apparent specific activity (S) when [RNA]: protein = 0* (arbitrary units)	[RNA]: protein (R) when apparent specific activity is zero	$10^4\ R/S$
LIVER	745	0.185	2.49
SPLEEN	455	0.115	2.52
KIDNEY	240	0.060	2.50

* Obtained by extrapolation: hypothetical value unless all membrane bound RNA is ribosomal RNA.

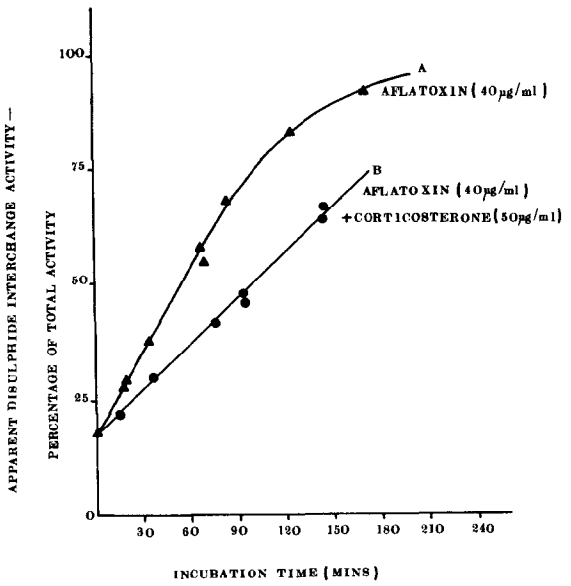

Fig. 2. Activation of rat-liver microsomes by aflatoxin B_1. —▲— A; incubation mixture contained aflatoxin B_1 (40 μg/ml), microsomes (approximately 4 mg/ml microsomal protein), sucrose (0.25 M), Tris (50 mM), KCl (25 mM) and $MgCl_2$ (5 mM) at pH 7.2. —●— B; as above but corticosterone (50 μg/ml) in addition. The apparent disulphide interchange activity (A') is shown as a percentage of the total, potential activity (A^0) for that particular preparation, obtained by applying the equation:

$$A^0 = A'/[1-\frac{Q}{(0.185)}]$$

where Q is the RNA:protein ratio (derived from fig. 1).

Microsomes were incubated at 25°C with aflatoxin B_1 (40 μg/ml) at a final concentration of approximately 4 mg (protein)/ml (for conditions see footnote to table 2) and samples periodically withdrawn for disulphide interchange enzyme assays. Untreated microsomes lose their disulphide interchange activity at a rate of approximately 10% per hour, whereas over the same period aflatoxin caused an activation equivalent to approximately 200% of the original value (fig. 2, plot A), continuing until all latent enzyme activity has been "unmasked". To verify the coupling of the increase in apparent enzyme activity with the removal of bound polysomes from the membranes, samples were prepared by incubating microsomes with aflatoxin B_1 (40 μg/ml, i.e. 12×10^{-5} M) at 5°C. They were layered over 2.0 M sucrose and centrifuged at 120,000 × g for 4 hours to remove unattached ribosomes, as described previously [4]. An estimation of the RNA:protein ratio of the interfacial material gives a measure of the number of ribosomes still bound to the membrane surface. It was found that incubation with aflatoxin caused a decrease in this ratio from 0.175 to 0.120. On the basis of the data summarised in table 1, this should give rise to an increase in apparent specific interchange activity equivalent to 190 units. The observed increase was 185–195 units confirming that aflatoxin B_1 displaces ribosomes from the membrane in complete units (cf. the stepwise detachment induced by EDTA) without modifying the actual interchange activity of the membrane. In an attempt to determine the effects of aflatoxin B_1 on the membrane-ribosome binding sites, the polysome-depleted membranes, produced after treatment of rat liver microsomes with aflatoxin B_1, were centrifuged over 2.0 M sucrose to remove unbound ribosomes and resuspended in buffered

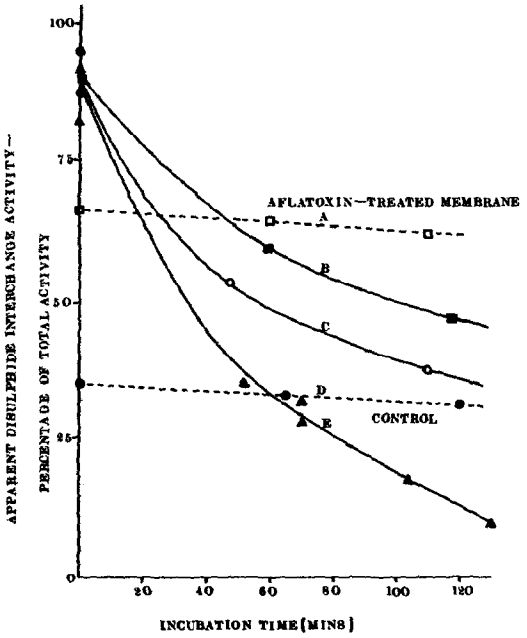

Fig. 3. The binding of polysomes to "polysome-depleted" membranes. All incubations contain sucrose (0.25 M), Tris (50 mM), KCl (25 mM), $MgCl_2$ (5 mM) and polysomes (1–2 mg RNA/ml, at pH 7.2. A (·—·□·—·), aflatoxin-treated membranes (approximately 4 mg/ml protein); B (—■—), "EDTA-treated" microsomes (approximately 4 mg/ml protein) plus cortisol (50 μg/ml); C (—○—), "EDTA-treated" microsomes; D (- - -●- - -). Untreated membranes; E (—▲—), "EDTA-treated" microsomes and corticosterone (50 μg/ml).

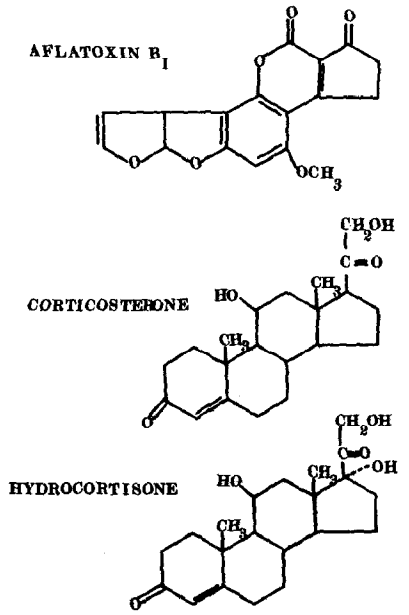

Fig. 4. Aflatoxin B_1 and the two steroid hormones used in this study.

$MgCl_2$ solution (5 mM) containing sucrose (0.25 M) and Tris (50 mM) at pH 7.5. Samples were similarly prepared after treatment of microsomes with 5 mM EDTA. The membrane preparations were incubated with a preparation of rat-liver polysomes at 25°C and the binding of polysomes to the "depleted" membranes was followed as a function of time by the decrease in the apparent interchange activity and the results are summarised in fig. 3. It is evident that the apparent activity of the aflatoxin-treated membranes (plot A) decays only at the same rate as the control (untreated membranes - plot D), whereas the activity of the EDTA-treated membranes (plot C) falls rapidly to the level of untreated microsomes (plot D), indicating complete reconstitution with a half time of circa 25 min. Evidently EDTA-treated membrane regains its ability to bind polysomes in the presence of 5 mM Mg^{++}, consistent with the findings of Süss et al. [8]. The aflatoxin-treated membranes, however, do not regain their ability to bind polysomes even after the removal of excess aflatoxin.

In view of the striking similarity between aflatoxin B_1 and the steroid hormones (fig. 4), the effect of some of these materials on the rate of attack by aflatoxin B_1 was determined. As shown in fig. 2, plot B, corticosterone (50 μg/ml) antagonises the effect of aflatoxin whereas cortisol has little obvious effect. The steroid competition effectively reduces the rate of aflatoxin attack but does not lessen the extent of the reaction. Some of the data obtained are summarised in table 2. Thus corticosterone competes with aflatoxin B_1 at a site on the membrane responsible, directly or indirectly, for polysome binding. The further inference that a steroid normally occupies a site on the membrane, thus enhancing polysome-membrane association, possibly by activating a "binding protein", is being further investigated. This idea is supported by the results of an experiment, which is illustrated in fig. 3 (plots B, C and E), showing the effects of cortisol and corticosterone on the rates of

Table 2

Incubation mixture*		Relative rates+ of activation	No. of determinations
Aflatoxin $\times 10^{-5}$ M	Corticosterone $\times 10^{-5}$ M		
12	0	100	9
9	0	79	2
6	0	58	4
3	0	35	2
12	15	60	5
12	30	30	2

* Incubation mixture also included microsomes (approximately 4 mgs/ml), sucrose (0.25 M), $MgCl_2$ (5 mM), Tris (50 mM), and KCl (25 mM) at pH 7.2. All incubations carried out at 25°C.
+ Results reproducible within circa ± 3%.

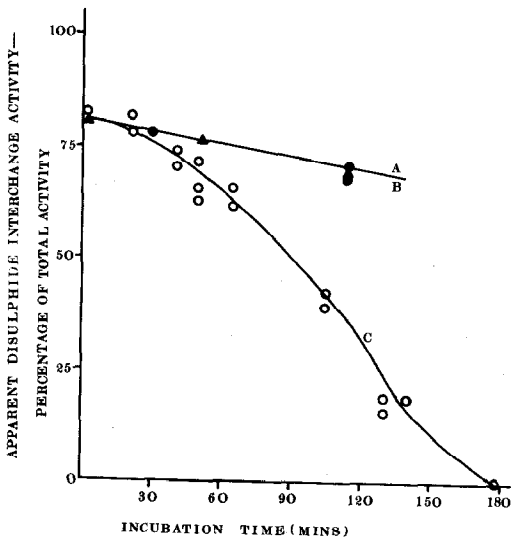

Fig. 5. The effect of corticosterone on the polysome-binding capacity of "smooth" microsomes. All incubations include "smooth", untreated microsomes (approximately 4 mg/ml protein), sucrose (0.25 M), Tris (50 mM), KCl (25 mM), $MgCl_2$ (5 mM) and in addition: A (–•–), polysome preparation (1–2 mg RNA/ml); B (–▲–), corticosterone (50 μg/ml); and C (–○–), polysomes (1–2 mg RNA/ml) and corticosterone (50 μg/ml).

reconstitution of "polysome-depleted" membrane and polysomes. It is clear that cortisol decreases the rate of polysome binding but the effect is small. In sharp contrast, corticosterone increases not only the rate but also the extent of polysome binding. The "extra" polysomes are presumably bound at sites which cannot be occupied in the absence of the hormone. This effect has been completely confirmed using "smooth" microsomal preparations as shown in fig. 5. The decay of apparent disulphide interchange activity of "smooth" membranes is very small in the presence of either corticosterone or polysomes (fig. 4, A and B) but rapid and complete (fig. 4, C) if the membrane preparation is incubated with a mixture of polysomes and the steroid. This suggests that corticosterone has caused the formation on the membrane of binding sites for polysomes and that each of these sites is associated with disulphide interchange activity. Since the effects of aflatoxin B_1 are on the membrane and not the polysomes and corticosterone and aflatoxin B_1 appear to compete, it is reasonable to suppose that corticosterone also interacts with the membrane. Our data can be explained by a simple hypothesis that a steroid hormone, related to corticosterone, can occupy specific sites on the membrane and that, when these sites are occupied, polysome binding can occur. The difference between "rough" and "smooth" endoplasmic reticulum would then be that the specific sites of the former are occupied by hormone, whereas those of the latter are not.

The action of aflatoxin B_1 is of interest since by displacing polysomes from the membrane, it could affect messenger stability. Whether or not its carcinogenic properties are related to effects of this sort remains to be investigated. It is important to note that this work has been carried out almost exclusively

on rat liver. It clearly needs to be extended to other tissues and the hormonal specificity of the binding process is under investigation.

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