The neoplastic cell

The effects of steroid hormones and carcinogens on the interaction of membranes with polysomes

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Protein synthesis in the cell involves particulate elements called ribosomes which translate the information coded as specific nucleotide sequences in messenger RNA molecules into amino acid sequences of proteins (Lambourg and Zamecnik, 1960). Ribosomes are frequently found in the cell in the form of aggregates called polysomes. The ribosomes in a polysome are believed to be attached to a single messenger RNA molecule and simultaneously to translate the encoded information to give polypeptides with a specific amino acid sequence (Howell, Loeb, and Tomkins, 1964). The basic mechanisms of the control of protein synthesis at the level of translation are not well understood.

Polysomes can be observed by electron microscopy to exist in the cell in two distinctive forms—free polysomes, unattached to membranes and membrane-bound polysomes (Palade, 1955; Teng and Hamilton, 1967). The membrane-polysome complex constitutes the 'rough' membranes of the endoplasmic reticulum. A variety of other membranes also occur in the cell, including 'smooth' endoplasmic reticulum, characterized by the absence of bound polysomes.

Accumulating evidence indicates that free and bound polysomes are probably involved in the biosynthesis of different classes of proteins (Siekevitz and Palade, 1960; Campbell, 1970). For example, it is probable that proteins exported from the cell are biosynthesized by membrane-bound polysomes (Siekevitz and Palade, 1960) and this may also be true of other important classes of protein such as those whose biosynthesis is induced by steroid hormones (Cox and Mathias, 1969). Clearly the control of the interaction of polysomes with membranes could be of profound importance in dictating the tissue-specific pattern of protein biosynthesis which characterizes the processes of differentiation and development. Indeed there are indications that the ratio of free to bound polysomes is a function of

the hormonal status of the organism (Cox and Mathias, 1969; Rancourt and Litwack, 1968; Mills and Topper, 1969; Maggi, Steggle, and Gahan, 1970). If chemical carcinogenesis is viewed as essentially an aberrant form of the processes of differentiation it is obviously important to investigate the effects of carcinogens on the interaction of membranes with polysomes.

The problem of investigating membrane-polysome interactions is considerably simplified by the fact that an enzyme present in the reticular membranes—which we shall call the disulphide rearranging enzyme (or 'rearrangase')—has its activity masked when polysomes are bound to the membranes and its activity is fully exposed when the polysomes are removed. Thus the activity of 'rearrangase' provides a relatively simple means of measuring quantitatively polysome attachment to membranes *in vitro*. The enzyme is present in both 'rough' and 'smooth' membrane fractions, but the activity in rough membranes is zero and only appears when the polysomes are removed (Williams, Gurari, and Rabin, 1968). 'Rearrangase' catalyses a complex reaction of disulphide exchange and is probably involved in the terminal stages of protein biosynthesis (Goldberger, Epstein, and Anfinsen, 1963).

Native bovine pancreatic ribonuclease contains four disulphide bonds. By reduction and reoxidation of the enzyme under denaturing conditions, it is possible to produce a protein in which the disulphide bonds are 'wrongly' paired and which is devoid of any catalytic action. If this 'randomly oxidized' material is used as substrate, the activity of 'rearrangase' can be measured by following the appearance of ribonuclease activity since the membrane-bound 'rearrangase' catalyses the 're-shuffling' of the sulphurs of the wrongly folded protein to give the native structure (Goldberger *et al*, 1963).

In fig 1 the specific activity of 'rearrangase' is plotted against the RNA/protein ratio of various

membrane subfractions prepared by differential centrifugation techniques from male rat liver. Similar and parallel plots are obtained from other tissues of the rat (Williams and Rabin, 1969). The RNA/protein ratio is a measure of the number of ribosomes bound per unit weight of the membrane. The linear relationship is not surprising, since the various subfractions are composed of rough and smooth membrane components but in different ratios. Notice that the 'roughest' components are totally devoid of enzymic activity. If those fractions with a high RNA/protein ratio are dialysed against EDTA, which causes polysome dissociation from the membranes, and the specific activity of the separated membranes is determined, the values obtained approach that corresponding to the intersection of the line in fig 1 with the ordinate axis. The degranulated rough membranes have approximately the same specific activity as smooth membranes: the 'enzyme density' is the same for all membranes from a given tissue and the enzyme is uniquely coupled to the loci of polysome binding. It may indeed be located in the vicinity of the ribosome-binding site which would be a favourable location for the enzyme to operate in the terminal stages of protein biosynthesis using the newly formed nascent protein as substrate.

Figure 2 illustrates an experiment in which liver

membranes were stripped of polysomes by EDTA treatment so as to uncover nearly all latent 'rearrangase' activity and the resultant degranulated membranes were incubated with fresh polysomes in the presence of magnesium ions. The 'rearrangase' activity falls to the initial low value of the original, mainly rough membranes as the polysomes reattach to the membranes. Notice that the experimental curve does not cross below that of the control of the unstripped membranes incubated with polysomes. Now the activity present in these unstripped membranes is due to the presence of smooth membranes and it is clear that although degranulated rough membranes will rebind polysomes, smooth membranes will not. This lack of interaction has been confirmed by using purified smooth membrane preparations. The reattachment of polysomes to stripped rough membranes has also been confirmed by measuring directly RNA bound to the membranes after separation of unbound polysomes by centrifugation (Williams and Rabin, 1969). Clearly smooth membranes are operationally different from degranulated rough membranes: the difference seems to be due to a component present in rough but absent in smooth reticulum as we shall see later.

The reattachment of polysomes to degranulated rough membranes is remarkably sex specific (fig 3). It can be seen that degranulated rough membranes

Fig 1

Fig 1 The relationship between apparent disulphide interchange activity (arbitrary units) and the RNA/protein ratio, for microsomal fractions derived from rat liver by differential centrifugation (assay methods as in Williams et al, 1968).

Fig 2

Fig 2 The binding of polysomes to 'polysome-depleted' membranes, monitored by the masking of disulphide interchange activity. Rough microsomal membranes were degranulated by EDTA (Williams et al, 1968; Williams and Rabin, 1969), isolated, and then incubated (~4 mg/ml protein) with excess free polysomes (~1 mg/ml RNA) at 25°C (O). Untreated rough membranes were also incubated with free polysomes as a control (●).

Fig. 3 The sex-specificity of association in vitro between free polysomes and degranulated rough membranes from rat liver. Conditions as described in legend of figure 2.

will re-bind polysomes from the same, but not the opposite sex. Before considering this sex specificity in detail we shall consider the nature of the differences between smooth and degranulated rough membranes. It is possible to prepare an ethyl acetate extract of male rough membranes which will activate both male and female smooth membranes to bind polysomes of either sex. A similar extract from male polysomes will promote the binding of female polysomes to female smooth membranes but not male polysomes to male smooth membranes. Analogous extracts from female polysomes promote the attachment of male polysomes to male membranes. These data are summarized in table I.

Although the presentation is qualitative, experimental determinations are actually very clear cut, and binding in the experimental systems seems to be almost an all-or-none phenomenon. The male polysomes contain a material which will activate the female but not the male system and vice versa. Male rough reticulum contains the components required to activate both the male and female systems. These unknown substances can be provided by the polysomes without extraction since a mixture of male plus female polysomes, but not the separate components, will bind to either male or female smooth membranes.

The above experiments strongly suggest that in

Smooth Membrane	Polysomes	Ethyl Acetate Extract	Formation of Membrane-polysome Complex
Male	Male	None	—
Male	Male	Male polysomes	—
Male	Male	Female polysomes	—
Male	Male	Male rough membranes	++
Male	Female	None	—
Female	Female	None	—
Female	Female	Female polysomes	—
Female	Female	Male polysomes	++
Female	Female	Male rough membranes	++
Female	Male	None	—
Female	Male + female	None	++
Male	Male + female	None	++

Table I Interaction of polysomes with smooth membranes as measured by disulphide rearranging enzyme

Membranes (5-7 mg protein/ml) were incubated with polysomes (2-5 mg RNA per ml) in the presence and absence of various 'ethyl acetate extracts'. The extracts were prepared by suspending the polysomes rough membranes in water (3 ml) and extracting three times with 6 ml portions of ice-cold ethyl acetate. The pooled extracts were evaporated to dryness, dissolved in 0.05 ml dimethyl formamide and diluted with 0.15 ml of 0.25 M sucrose in 50 mM Tris-chloride containing 25 mM KCL and 5 mM MgCl₂ (TKM).

Fig 4 The sex-specific effects of steroid hormones in promoting association of free polysomes and smooth microsomal membranes *in vitro*. Incubations of steroids with male and female smooth microsomes and polysomes. No steroid present (●); in presence of $2 \mu\text{g ml}^{-1}$ oestradiol (▲); in presence of $2 \mu\text{g ml}^{-1}$ testosterone (Δ).

male rat liver, smooth membranes will not bind polysomes because there is a deficiency of a material which is soluble in organic solvents and present in female polysomes or male rough endoplasmic reticulum. In the female there would be an analogous deficiency, but of a different material which is present in male polysomes. One obvious possibility would be the involvement of the pair of sex hormones, with oestradiol being deficient in the male and testosterone deficient in the female. This would require that testosterone or oestradiol is present in male or female polysomes, respectively, as isolated. These hormones have, indeed, been previously implicated in the interaction of smooth membranes with polysomes (Rabin, Sunshine, and Williams, 1970; Sunshine, Williams, and Rabin, 1971) and some of the experimental evidence is presented in figure 4. It is clearly demonstrated in fig 4 that oestradiol promotes the binding of male polysomes to male smooth membranes. Testosterone is without any effect. The binding in the presence of oestradiol *in vitro* has been confirmed by electron microscopy (James, Rabin, and Williams, 1969) and it has been estimated, very approximately, that one molecule of steroid hormone is required per ribosome bound to the membrane. Figure 4 illustrates the situation in the female: the effects are totally opposite to the male. Testosterone promotes polysome binding, oestradiol is without effect. The concentrations of hormone required are again of the order of one molecule per bound ribosome. These experiments show very clear-cut differences between male and female animals, which could derive from sex specificity of steroid interactions with membranes and/or polysomes.

One, but by no means the only, possible explanation of the results is (1) that in both sexes oestradiol and testosterone are required to be simultaneously present to activate the membranes for polysome binding; (2) that polysomes and/or membranes of the female already contain oestradiol, or an equivalent material, and those of the male a substance with equivalent properties to testosterone. This explanation would, of course, be entirely consistent with the ability of the ethyl acetate extracts of polysomes to promote membrane-polysome interaction in the opposite sex. To shed further light on this problem some experiments were conducted to determine the steroid hormone requirements for the interaction of membranes with polysomes from the opposite sex. Some of these results are summarized in tables II and III. It can be seen that testosterone promotes the interaction of male membranes with female polysomes and oestradiol a similar interaction of female membranes with male polysomes.

It seems clear from the work described above that steroid hormones (or equivalent materials extracted from polysomes or membranes) are required for membrane-polysome interactions to occur. The requirements are sex-specific and are for oestradiol (or equivalent material extracted from female polysomes) in the male and testosterone (or equivalent material extracted from male polysomes) in the female. The crossed-sex experiments suggest there may also be a requirement for the other hormone, although this possibility cannot yet be tested experimentally in the interaction of membranes with polysomes from the same sex, because the male and female polysomes already contain, respectively,

Polysome Sex	Added Steroid	Formation of Membrane-polysome Complex
Female	None	—
Female	Oestradiol	—
Female	Testosterone	+
Male	None	—
Male	Testosterone	—
Male	Oestradiol	+

Table II Steroid hormone requirements for the interaction of polysomes with female smooth membranes¹

¹Incubations of membranes and polysomes, and assay for complex formation as in table I

Polysome Sex	Added Steroid	Formation of Membrane-polysome Complex
Male	None	—
Male	Testosterone	—
Male	Oestradiol	+
Female	None	—
Female	Oestradiol	—
Female	Testosterone	+

Table III Steroid hormone requirements for the interaction of polysome with male smooth membranes¹

¹Incubations of membranes and polysomes, and assay for complex formation as in table I

substances equivalent to testosterone and oestradiol. It is certainly true to say that in all systems in which polysomes interact with membranes both hormones (or equivalent materials) are present. We are at present attempting to deplete polysomes of their hormone content to test whether both hormones are involved in the binding process.

Further relevant information has been obtained by direct binding studies of steroid hormones to membranes (Blyth, Freedman, and Rabin, 1971). Figure 5 shows the binding curves obtained for the interaction of oestradiol with male smooth and degranulated rough membranes. There are some quite profound differences between the two types of membrane. The smooth membranes have sites at which the hormone is bound very tightly (region A-B, we shall call these 'tight sites') and which can be saturated; at higher hormone concentration (region B-C) there is an almost linear partitioning of the hormone between the membranes and solution. At still higher hormone concentration (region C-D) a further set of hormone-binding sites comes into operation. Degranulated rough membranes do not possess sites in the regions A-B and C-D. We have evidence that binding in the region C-D involves the microsomal hydroxylase complex, which is mainly located in smooth membranes. We shall consider only binding in the region A-B. The binding curves of the 'tight sites' only are shown in

Fig 5 Direct titration of ³H-oestradiol with male rat liver microsomes. Methods as in Blyth et al (1971). Smooth microsomes (■); EDTA-treated smooth microsomes (●); EDTA degranulated rough microsomes (○).

figures 6 and 7. They show the same remarkable sex specificity as the interaction of smooth membranes with polysomes from the same sex. It can be seen that male smooth membranes possess 'tight sites' for oestradiol and female membranes similar sites for testosterone. The sites are very specific since oestradiol and testosterone do not compete with each other for these 'tight sites'. Degranulated membranes from either sex apparently do not possess 'tight sites' for either hormone.

Degranulated membranes possess high affinity binding sites for oestradiol and testosterone in the male and female, respectively. These sites are not observed in titration experiments of the type described above because they are already occupied by the appropriate steroid (Blyth, Cooper, Roobol, and Rabin, 1972). It is presumably for this reason that degranulated membranes interact with ribosomes of the same sex without the necessity for the addition of a hormone.

We have also investigated the effects of a range of carcinogenic and other substances on the interaction of membranes with steroids and polysomes. Our earlier experiments showed that the hepatotoxin and carcinogen, aflatoxin B₁, will directly degranulate the rough endoplasmic reticulum by irreversible interaction with the membranes (Williams and Rabin, 1969). Figure 8 shows the degranulating effect for both male and female

Fig 6

Fig 6 Titration of the 'tight' binding sites (Blyth et al, 1971) for oestradiol in male (Δ) and female ($\bullet$) rat liver smooth microsomes.

Fig 7

Fig 7 Titration of the 'tight' binding sites (Blyth et al, 1971) for testosterone in male (∇) and female ($\bullet$) rat liver smooth microsomes.

membranes as measured by assaying the 're-arrangase' enzyme. The results have been confirmed by direct measurements of RNA attached to the membranes (Williams and Rabin, 1969). If the carcinogen is acting by interfering with a site on the membrane which binds steroid hormones, then it is

to be expected that the appropriate hormone will protect the membrane against the degranulating effects of the carcinogen. This is indeed observed, as shown in fig 8 and, again, the protection is sex specific: oestradiol protects male and testosterone female membranes. Aflatoxin B_1 also destroys the

Fig 8 The effects of steroid hormones on the reaction of aflatoxin B_1 with rough microsomes from male and female rat liver. Membranes at ~ 4 mg/ml protein. Control incubation (Δ); incubation with aflatoxin B_1 (40 μ g/ml) alone ($\blacktriangle$); incubation with aflatoxin B_1 and oestradiol (10 μ g/ml) ($\circ$); incubation with aflatoxin B_1 and testosterone (10 μ g/ml) ($\bullet$).

ability of smooth membranes to interact with polysomes in the presence of the appropriate hormone. These effects of aflatoxin B₁ are also observed by feeding experiments *in vivo*. The smooth membranes from male rats fed on a diet containing 5 ppm aflatoxin B₁ have totally lost their ability to bind polysomes in the presence of oestradiol in just two days. The carcinogen destroys the ability of smooth membranes to react with polysomes much more rapidly than it degranulates rough membranes.

Binding studies have demonstrated that aflatoxin B₁ destroys the tight binding sites for steroid

hormones on smooth membranes and some of the relevant experiments are illustrated in figure 9. It can be seen that oestradiol, but not testosterone, protects the tight oestradiol sites on male smooth membranes. Similarly, testosterone, but not oestradiol, protects the sites for this hormone on female membranes.

To summarize, the data presented show that oestradiol and testosterone respectively activated male and female smooth membranes to bind polysomes. There are specific 'tight sites' for oestradiol on male and for testosterone on female smooth membranes. These sites are destroyed irreversibly by aflatoxin B₁. These hormones, or equivalent substances, are already present in rough reticulum. Aflatoxin B₁ causes the irreversible displacement of polysomes from rough membranes and the evidence is consistent with the blocking of a specific hormone site. The steroid sites on both rough and smooth membrane can be specifically protected by the hormone involved against destruction by the carcinogen.

A number of other carcinogenic substances have shown to produce the same effects as aflatoxin B₁. Table IV shows the relative degranulation, measured as increase in 'rearrangase' enzyme activity, caused by incubation of microsomal membranes, mainly rough but containing some smooth, with various carcinogenic compounds and some innocuous relatives, in the presence of 1 mM NADPH (and postmitochondrial supernatant). Clearly some of the compounds do indeed cause degranulation and in

Fig. 9 The effect of aflatoxin B₁ on 'tight' steroid-binding sites (Blyth et al, 1971) of smooth microsomes from rat liver. Titration of male microsomes with ³H-oestradiol. Titration of female microsomes with ¹⁴C-testosterone. For both graphs: untreated membranes (○); membranes (3 mg/ml protein) incubated at 25°C with aflatoxin B₁ (3 μM) for 20 hours before titration (●); membranes incubated with aflatoxin, as above, in presence of oestradiol (375 nM) (□); membranes incubated with aflatoxin, as above, in presence of testosterone (375 nM) (■).

Substance Tested ¹	Rate of Enzyme Activation ²	Carcinogenicity
Naphthalene	0	Nil
2-Naphthylamine	0-3	±
Anthracene	0	Nil
2,7-Diaminofluorene	8	+
1,2-Benzanthracene	5	+
1,2-Benzanthraquinone	8	+
2-Aminochrysene	8	+
6-Aminochrysene	0	Nil
3-Aminopyrene	9	+
3,4-Benzpyrene	20	+
Benzo(b)chrysene	0	Nil
3,4,8,9-Dibenzopyrene	13	+
Carbon tetrachloride (15 μg ml ⁻¹)		
Aflatoxin B ₁ (10 μg ml ⁻¹) ³	10	+

Table IV Rate of increase of apparent disulphide interchange enzyme activity of microsomal membranes from rat liver on incubation with the substances indicated in the presence of NADPH

¹Incubated at 20°C with microsomal membranes (~10 mg protein ml⁻¹) at a concentration of 5 μg ml⁻¹ (unless otherwise stated) in the presence of 1 mM NADPH, 50 mM tris, 25 mM KCl, 5 mM MgCl₂ and 250 mM sucrose, at pH 7.3. Postmicrosomal supernatant was added to 10% final volume.

²Percentage of total enzyme activity released per hour.

³Absence of NADPH

this series correlation with carcinogenicity is complete. The NADPH is an absolute requirement in each case. The term 'carcinogenicity' as applied to the active compounds in the table needs some definition. A compound is deemed 'carcinogenic' if it can cause tumour formation in any tissue in the rat; clearly not all of the compounds listed in the table are hepatocarcinogens.

To confirm that the apparent enzyme activation was the result of degranulation, a selection of the compounds, listed in table IV, were incubated with microsomal membranes in the presence of NADPH. The incubates were then centrifuged at 120 000 *g* for four hours over a layer of 2.0M sucrose to separate membranes from unbound ribosomes. The difference in RNA/protein ratios of the membranes, floating on the 2.0M sucrose layer, compared with that of untreated membrane gives a direct measure, in terms of RNA, of the degranulation. Table V summarizes the results obtained which clearly correlate well with the results obtained indirectly in table IV. The carcinogen-pretreated membranes would not bind added fresh ribosomes in the presence of oestradiol ($1\mu\text{g ml}^{-1}$) even after extensive washing, so that not only is the degranulation operationally irreversible, but all of the potential ribosome-binding sites on the original smooth membranes are destroyed as well.

The mechanism of the carcinogen activation and subsequent degranulation is not yet clear, but studies with various modifiers of the microsomal hydroxylase complex, summarized in table VI, suggest the involvement of the cytochrome P450-flavoprotein enzyme system present in microsomal membranes (Orrenius, 1965). The absolute requirement for NADPH, the partial inhibition by alternative substrates, and the complete inhibition by CO and the P450 specific inhibitor—SKF 525A—all point to this conclusion. Similarly the complete inhibition of the overall degranulation by low concentrations of oestradiol strongly suggest that it is the steroid-specific site, responsible for maintaining

Substance Tested ¹	RNA Removed from Membrane (% control)		
	Measured Directly	Calculated ²	
Naphthalene	0	0	Nil
Anthracene	0	0	Nil
2,7-Diaminofluorene	30	30	+
2-Aminochrysene	30	30	+
6-Aminochrysene	0	0	Nil
3-Aminopyrene	27	35	+
3,4-Benzpyrene	25	40	+
Carbon tetrachloride ($15\mu\text{g ml}^{-1}$)	35	30	+

Table V Degranulation of rough surfaced microsomal membranes from male rat liver caused by incubation with the substance indicated in the presence of NADPH

¹Mixture of rough and smooth membranes incubated at 20°C for 2 hours as described in footnote to table IV, and centrifuged over a layer of 2.0 M sucrose for 4 hours, 120 000 *g* to remove unbound ribosomes.

²Corresponding values calculated from the data summarized in table IV.

membrane-polysome complex integrity; this is attacked by the activated 'carcinogen'.

The phenomena observed *in vitro* for aflatoxin are also observed *in vivo*. Substantial degranulation of rough reticular membranes occurs on feeding the toxin at a level of 5 ppm for nine weeks. All of the potential ribosome-binding sites on the smooth membranes are destroyed on feeding the toxin for just two days at the same level. *In vivo* experiments with another carcinogen, ethionine (Williams, Rabin, and Kisilevsky, 1972), also showed substantial degranulation of rough reticular membranes. Unlike aflatoxin, ethionine produces a lesion in the ribosomes which makes them incapable of interacting with membranes from control animals. The membranes from ethionine-treated animals still retain ribosome-binding sites and will interact, under appropriate conditions, with ribosomes from control animals. Ethionine is the only compound discovered so far in which the ribosomes, rather than the membranes, are damaged by a carcinogen.

Incubation	Rates of Degranulation ^a		
	Benzantraquinone	Dibenzpyrene	2-Aminochrysene
Control	8	13	8
- NADPH	0	0	0
+ Naphthalene ($10\mu\text{g ml}^{-1}$)	2	2	2
+ CO	0	0	Not tested
+ SKF 525A (1 mM)	0	0	0
+ Oestradiol ($1\mu\text{g ml}^{-1}$)	0.5	0	0.5
+ Testosterone ($1\mu\text{g ml}^{-1}$)	Not tested	9	Not tested

Table VI effect of various substances on the carcinogen-induced degranulation of microsomal membranes from male rat liver

¹Conditions exactly as described for table IV except additions where indicated.

²Measured as the rate of increase of apparent disulphide interchange enzyme activity.

The importance of this type of interaction in the transformation of tissues *in vivo* is clearly impossible to evaluate at the present time but such an initial interaction *in vivo* is certainly consistent with the frequent reports of rough endoplasmic membrane degranulation (Baglio and Farber, 1965; Svoboda and Higginson, 1968; Flaks, 1970) and binding of carcinogens to cytoplasmic and microsomal protein (Miller and Miller, 1952; Wiest and Heidelberger, 1953; Hultin, 1956) during chemical tumorigenesis.

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