ENDOPLASMIC MEMBRANE DEGRANULATION *IN VIVO* AS A RESULT OF ETHIONINE INTOXICATION

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1. Introduction

The effects of ethionine on hepatic protein synthesis appear to be accompanied by extensive degranulation of the rough surfaced membranes of the endoplasmic reticulum, the biochemical basis of which is not understood [1, 2]. In this report we demonstrate that the basic lesion which leads to damage of the rough membranes is in the ribosomes. Previous work in this laboratory has provided evidence for the existence of steroid-dependent ribosome binding sites on the endoplasmic membranes [3]. The ribosome binding sites are closely associated with the loci of activity of an enzyme catalysing disulphide interchange, in such a way that the activity is masked by bound ribosomes [4]. Hence, by measuring the disulphide interchange enzyme activity, it is possible to monitor changes in the association of ribosomes with membrane *in vitro* [4–6]. This technique was used to investigate the disturbed pattern of ribosome binding to endoplasmic membranes that appears to result from ethionine intoxication.

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2. Materials and methods

Ribosome-membrane association was followed *in vitro* indirectly by monitoring apparent disulphide interchange enzyme activity as described previously [4]. All solutions were made up in a buffer containing 50 mM Tris base, 25 mM KCl and 5 mM $MgCl_2$, titrated to pH 7.5 with HCl (TKM). Protein was estimated by the method described by Lowry et al. [7], RNA by the method of Schmidt and Thannhauser [8], using the extinction co-efficient for hydrolysed RNA quoted by Fleck and Begg [9], and cholesterol by the technique of Abell et al. [10].

3. Results and discussion

Adult, female albino rats (body weight 175–185 g) of the Porton strain received ethionine at a dose of 1 mg/g body weight and were sacrificed 4 hr later by cervical fracture. The livers were rapidly removed into ice-cold buffer (TKM) containing 0.25 M sucrose. After shredding, the livers were homogenised in 3–4 volumes of the same buffer and the resulting homogenate centrifuged at 19,000 *g* for 15–20 min. The post-mitochondrial supernatant was centrifuged, after layering over 2.0 M sucrose in TKM, at 120,000

g for ca. 3 hr as described previously to separate microsomal membranes and membrane-free ribosomes [3]. The membrane fraction floating on the 2.0 M sucrose layer after centrifugation contained all membrane bound RNA. All other ribosomal RNA either pellets or remains dispersed in the 2.0 M sucrose. Table 1 shows the recovery of RNA in these various

Table 1

RNA: protein ratios of total microsomal membrane preparations and the distribution of RNA within microsomal subfractions of liver from control and ethionine treated (1 mg/g body weight) rats after 4 hr, expressed as a percentage of total microsomal RNA.

Fraction	Control	Treated
RNA: protein (μg/mg membrane protein) of membrane fraction	93	54
Percentage total RNA* recovered as:		
Membrane RNA	55	21
Slowly sedimenting, non-membrane RNA**	19	56
Peleted RNA	26	23

* Values are averages of four animals each control and treated (Individual variation < ± 10%).

** RNA remaining in the 2.0 M sucrose layer after centrifugation.

fractions, prepared from ethionine treated animals and controls of the same age and weight. The proportion of total RNA recovered found in the pellet is clearly the same in both cases but there is a large decrease in membrane bound RNA in tissue from treated animals. This just corresponds to the increase in slowly sedimenting ribosomes, i.e. those which remain in the 2.0 M sucrose layer and do not pellet during centrifugation. These data are clearly consistent with the observed degranulation *in vivo* [1] and the increase in the proportion of small polysomes and monomeric ribosomes [11] during ethionine intoxication. The exact correspondence between the loss of RNA from the membrane fraction and the increase in the slowly sedimenting RNA fraction suggests the possibility that the extra RNA in the latter fraction derives from polysomes removed from the membrane.

The level of cholesterol in smooth surfaced membrane is characteristically high compared with that of rough fractions, containing a large number of bound ribosomes ([12] and see table 2). Table 2 shows that

Table 2

Cholesterol and RNA content of hepatic microsomal preparations from control and ethionine treated rats.

Fraction		RNA/protein (μg/mg membrane protein)	Cholesterol/protein (μg/mg membrane protein)
Total microsomal membrane preparation (Control)		93	19
Total microsomal membrane preparation (Treated)		54	18.5
Smooth surfaced subfraction	Control	30	27
Rough surfaced subfraction	Control	175	5

the decrease in bound RNA found in ethionine treated animals is not accompanied by any detectable increase in cholesterol content of the microsomal membrane preparation. This finding clearly supports the view that the decrease in RNA content of microsomal membranes from treated animals is due to direct membrane degranulation and not to an increase in proportion of smooth membranes, often observed as a response to drug challenge. In order to test this hypothesis further, the ability of ribosomes from control and treated animals to bind to membranes from both sources was determined. It has been demonstrated that ribosomes mask the activity of the disulphide interchage enzyme when bound to the membrane [3] and this has been used to monitor the association of ribosomes with membranes *in vitro* [4–6].

Enzyme activity is apparent in smooth surfaced membrane subfractions as prepared but not in rough. However, treatment with a chelating agent, removing ribosomes from the surface of the rough membrane, uncovers latent activity which can be "remasked" by adding back ribosomes in the presence of Mg^{2+} [5]. The latent activity of the rough membranes is of the same magnitude as that apparent in smooth [3, 5]. Further, the activity of smooth membranes decreases in the presence of a polysome preparation if an appro-

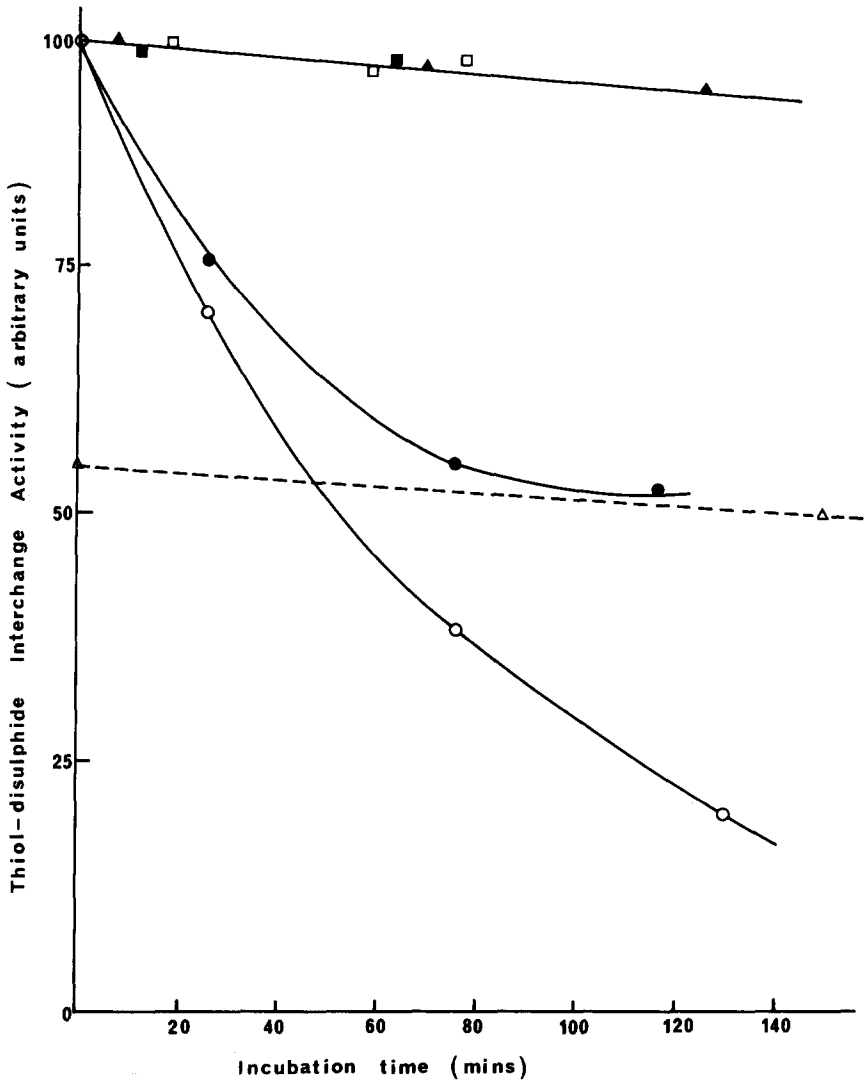

Fig. 1. The change in apparent disulphide interchange enzyme activity of microsomal membranes when incubated with a polysome preparation in the presence and absence of testosterone. Microsomal membranes from treated animals (10 mg protein/ml) were incubated with a polysome preparation from control animals, 5 mg RNA/ml, in the presence of testosterone, 2 μg/ml, (○–○–○), or in its absence (●–●–●). Membranes from control (△–△–△) and treated (□–□–□) animals were incubated alone. Membranes and ribosomes both from treated animals were also incubated together in the presence (▲–▲–▲) or absence (■–■–■) of the steroid. All incubations were carried out at 20° in TKM containing 0.25 M sucrose.

priate steroid is added, suggesting steroid dependent "potential" ribosome binding sites in the smooth. The steroid requirement is quite sex specific–membranes from male animals requiring oestradiol and those from female animals testosterone. These data suggest that ribosome binding sites are distributed equally over both rough and smooth surfaced endoplasmic reticulum – the sites in rough being active and those in smooth requiring an added steroid hormone for activity.

Unpublished work in this laboratory strongly suggests that the sites in rough membrane already contain the steroid required by the smooth (C.A. Blyth and B.R. Rabin). Hence, membranes derived from both treated and control animals (female) were incubated with polysomes from the same animals in the presence or absence of testosterone. The results are shown in fig. 1. The activity of "treated" membrane, incubated

with a preparation of "free" ribosomes made from control livers, approaches zero in the presence of testosterone (2 μg/ml) showing that the membrane from intoxicated animals retains its ability to bind normal ribosomes. However if testosterone is omitted from the incubation mixture a decrease in activity is still seen, but it does not fall below the level of apparent enzyme activity shown by the "control" membranes. It is of interest to note that this situation is exactly analogous to that in which membranes stripped of ribosomes by EDTA are used [5] – it seems then that the membranes "degranulated" by ethionine differ from normal smooth membranes in that they can recombine with polysomes from untreated animals without steroid addition. The activity of membranes incubated with ribosomes from intoxicated animals decreases only at the rate of the control (without ribosomes) in the presence or absence of steroid.

The data presented here strongly suggest that ethionine treatment causes a degranulation of hepatic rough surfaced endoplasmic reticulum *in vivo* and does not simply induce the proliferation of smooth membrane. The lesion appears to be in the ribosomes and not in the membranes. Many other carcinogenic substances appear to cause similar degranulation both *in vivo* [2] and *in vitro* [6], although ethionine is the only case yet discovered in which the ribosomes rather than the membranes are damaged. Furthermore, *in vivo*, the liver toxins and carcinogens aflatoxin B_1, dimethyl nitrosamine, carbon tetrachloride, and ethionine all cause an inhibition of protein synthesis, an apparent degranulation of endoplasmic reticulum, and an apparent labilisation of polysomes [2, 11, 13–17]. The importance of such effects on protein synthesis and the endoplasmic reticulum during chemical carcinogenesis and intoxication remain to be evaluated. The significance of such interactions at the microsomal level is currently under investigation.

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