Studies on the degranulation test for carcinogens

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The radiometric assay of degranulation of the hepatic endoplasmic reticulum by chemical carcinogens has been re-examined. Both 1,2,3,4,- and 1,25,6-dibenzanthracenes caused degranulation of rough membranes in vitro; with acetamidofluorenes and naphthylamines the carcinogenic analogues caused moderately greater degranulation. Degranulation by 1,2,3,4-dibenzantracene was rapid and was maximal after 5 min incubation. Pretreatment of animals with phenobarbital or methylcholanthrene increased the fraction of rough membranes, but these were not fully granulated. The assay is limited in specificity and sensitivity because the 1.35 M sucrose gradient does not effectively separate rough and smooth membranes, and sedimented membranes are contaminated with aggregates of free ribosomes.

Degranulation *Endoplasmic reticulum* *Carcinogens* *Ribosomes* *Microsomes*

1. INTRODUCTION

The membranes of the hepatic endoplasmic reticulum are usually considered to be of two forms, namely, 'rough' (RER) and 'smooth' (SER), depending on whether or not they have bound ribosomes. Carcinogens cause degranulation of the RER in vitro [1] and this has been proposed as the basis of a screening test for chemical carcinogens [2]. However, predictions of the degranulation test showed poor correlation with known carcinogenicity [3]. We have re-examined the radiometric assay of degranulation [2], studied various factors that may influence the results, and shown that the sucrose gradient technique does not effectively separate SER and RER membranes.

2. METHODS

The degranulation assay in [2] was followed with minor alterations. Male Wistar-albino rats starved for 24 h prior to i.p. injection of [6-^{14}C]orotic acid (15 μCi) in saline, were allowed food, and killed by cervical dislocation 17 h later. A 30% (w/v) liver homogenate in 0.25 M STKM buffer (0.25 M sucrose in TKM buffer: 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–HCl, adjusted to pH 7.5 with 1 M HCl), was centrifuged at 115000 × $g_{av.}$ for 20 min to obtain the post-mitochondrial supernatant, which was layered over a discontinuous sucrose gradient of 5 ml of 2.0 M STKM buffer (2 M sucrose in TKM buffer) underlying 10 ml of 1.35 M STKM, in 25 ml polycarbonate tubes. The gradients were centrifuged at 115000 × $g_{av.}$ for 4 h, and membranes at the gradient interface were harvested using a syringe. The membranes were diluted with 0.25 M STKM and pelleted through 0.5 M STKM (0.5 M sucrose in TKM buffer) at 115000 × $g_{av.}$ for 1 h. The fractionated microsomes were resuspended in 0.25 M STKM to give a final concentration of about 10 mg/ml.

The incubation mixtures containing about 20 mg microsomal protein, 3 mM trisodium isocitrate, 500 mM NADP, 5 mM $MgSO_4$, 2.4 IU isocitrate dehydrogenase, 75 μl of a solution of the test compound (1 mg/ml) in DMSO, and 0.25 M STKM buffer in a final volume of 6.0 ml, were incubated for 2 h at 20°C. Incubations were stopped by rapid cooling in ice-water together with addition of 1.35 M STKM (5 ml) to bring the final sucrose concentration to 0.7 M. The incubation mixtures were layered over a gradient consisting of 6 ml 2.0 M STKM underlying 4 ml 1.35 M STKM in

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25 ml tubes, and were centrifuged at 70000 × $g_{av.}$ for 12 h. The membranes were removed from the boundary layer, diluted with 0.25 M STKM, pelleted at 115 000 × $g_{av.}$ for 1 h, and the microsomal pellets resuspended in deionised water by ultra-sonication.

Aliquots of the suspension were assayed for protein [4], and were mixed with Instagel (3 ml) for determination of ^{14}C in a LKB Ultrabeta LS counter. The specific activities of the control samples were determined and the difference between RER and SER was taken as 100%. The percentage degranulation of the test samples was then calculated.

3. RESULTS

Pairs of 3 carcinogenic chemicals and their non-carcinogenic analogues were used to evaluate the assay. The results (table 1) show that with the acetylaminofluorenes and the naphthylamines the carcinogenic analogue caused moderately greater degranulation, but this was not the case with the dibenzanthracenes. Variation of the incubation time using 1,2,3,4-dibenzanthracene as test compound (fig.1), showed that degranulation was essentially complete within the first 5 min, which indicates that degranulation is very rapid.

The solvent for the test carcinogens, DMSO resulted in about 19% degranulation, while in-

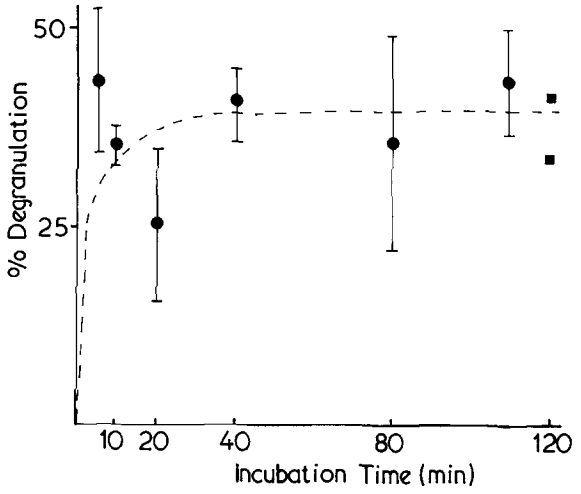

Fig.1. Effect of incubation time on the apparent degranulation of RER membranes by dibenzanthracene. Membranes were prepared as described in section 2, and were incubated with 1,2,3,4-dibenz(a)anthracene dissolved in DMSO. At various intervals, samples were taken and after centrifuging overnight on a sucrose gradient the membranes were assayed for protein and ^{14}C-activity. Samples of RER and SER membranes were also assayed, as controls, after incubation for a similar time without carcinogen. The difference in activity between the RER and SER controls represents 100% degranulation; the points shown (■) were obtained in 2 similar expts, and are included for comparison. Points shown are means of 4 determinations, vertical bars show SEM.

Table 1

Degranulation of RER membranes by paired (carcinogen/non-carcinogen) compounds determined by radiometric assay

Compound	% Degranulation	Known carcinogenicity
2-Acetylaminofluorene	25 ± 1 (8)	+
4-Acetylaminofluorene	17 ± 3 (8)	–
1,2,5,6-Dibenz(a,h)anthracene	23 ± 1 (12)	+
1,2,3,4-Dibenz(a,c)anthracene	33 ± 8 (12)	?
β-Naphthylamine	27 ± 2 (4)	+
α-Naphthylamine	14 ± 1 (4)	–

RER and SER membranes were prepared as described in the text. The compounds under test were dissolved in DMSO and added to a suspension of the RER membranes in STKM buffer. After 2 h incubation at 20°C, the degranulated membranes were harvested by centrifugation and assayed for protein and ^{14}C-activity. The percentage degranulation was calculated as described in section 2

Values shown are the mean ± SEM for the no. of expts shown (*n*)

cubation of the RER without DMSO, or storage in ice for the same time, resulted in an apparent increase of 30–45% in granulation. This confirms that DMSO may not be the ideal solvent for the test compounds (Fey, White and Rabin, personal communication), and also indicates that RER membrane fractions may become contaminated with free ribosomes. These ribosomes may form aggregates during the incubation which would cosediment with the membranes during the later separation. Smooth membranes incubated with dibenzanthracene showed a further loss (20%) of radioactivity (ribosomal RNA), which indicates that the SER fraction is not entirely smooth. It may therefore be expected that membrane fractions vary from totally smooth to totally rough, so that the gradient chosen for fractionation would affect the results obtained. A series of gradient separations using different sucrose concentrations were therefore obtained, and the protein at the interface and in the pellet was calculated as a proportion of the protein applied to the gradient. From these results, the distribution of microsomal membranes over the gradient density range studied was calculated. The results (fig.2) indicate that the majority of membranes have a density between 1.12–1.18 g/ml, and have ribosomes attached. The usual gradient step of 1.35 M sucrose (1.15 g/ml) would divide the membrane population into 'rougher' and 'smoother' fractions, but the smooth fraction would contain a substantial amount of granulated membranes. The overall difference between the two fractions would thus be less than originally supposed. Pretreatment of animals with the microsomal enzyme inducing agents, phenobarbitone or 3-methylcholanthrene, both result in an increase in the membrane fraction that would be separated as RER, but which is not fully granulated (fig.2).

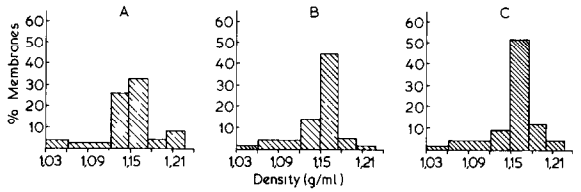

Fig.2. Density profile of rat liver microsomal isolated from treated and control animals. Male Wistar-albino rats were given a single i.p. injection of either corn-oil (A), 80 mg/kg phenobarbitone (B), or 25 mg/kg 3-methylcholanthrene (C). The animals were killed 24 h later by cervical dislocation and liver microsomal membranes prepared. The membranes were fractionated on a series of discontinuous sucrose gradients of varying molarity, and the recovery of microsomal protein at the gradient interface and in the pellet was calculated. From the recovery data, the percentage microsomal population having a density within a particular range was calculated. The results are shown as population distribution histograms.

4. DISCUSSION

These studies explain some of the variable results previously obtained using the degranulation assay for potential carcinogens. The apparently rapid degranulation could be an artifact, and the degree of degranulation may be affected by lipid peroxidation during the long assay procedure [5], or by the effect of prolonged centrifugation at high forces [6]. If the latter were the case, fractionation by the flotation method may prove less damaging than the sedimentation method. The period of incubation may also give rise to spurious results due to ribosomal aggregation [7,8].

Many studies of chemical-induced membrane degranulation have been carried out using membranes fractionated on a gradient of 1.35 M sucrose. It has now been shown that this gradient results in a separation of microsomal membranes into two fractions, the components of which are not very different from each other. Mailman [9] resolved this problem by using a less dense gradient (1.05 M sucrose) for the separation, and thus isolated the true 'smooth' fraction from the remainder. The use of a triple-interface discontinuous gradient of 2 M/1.5 M/1.1 M/0.25 M sucrose would offer even better preparations by allowing separation of true SER and RER from the remaining partially granulated membranes.

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