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ACTIVATION *IN VITRO* OF GLUCOSE-6-PHOSPHATASE, INORGANIC PYROPHOSPHATE–GLUCOSE PHOSPHOTRANSFERASE AND RELATED ENZYMES

RELATIONSHIP TO MICROSOMAL MEMBRANE STRUCTURE

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SUMMARY

1. The elevation in enzymatic activity of certain microsomal enzymes caused *in vitro* by treatment with NH_4OH or deoxycholate has been studied in relationship to the appearance of the membranes in corresponding electron micrographs.

2. When freshly prepared liver microsomes were treated at 0° with NH_4OH (about pH 9.8), activation of glucose-6-phosphatase (EC 3.1.3.9), inorganic pyrophosphatase, inorganic pyrophosphate–glucose phosphotransferase and inorganic pyrophosphate–glycerol phosphotransferase was not instantaneous.

3. Exposure to NH_4OH for 15 to 30 min at 30° or contact for many hours at 0° resulted in a maximum *in vitro* elevation of the enzyme activities that was approximately quantitatively equal to that observed instantaneously with such detergents as deoxycholate or Triton X-100.

4. An electron microscopic study of NH_4OH-activated microsomes at 0° showed that ribosomes were largely retained on the membranes when about 50 % of maximum stimulation of enzymatic activities occurred. Longer exposure at 0° or brief warming at 30° with NH_4OH, effecting full enzymatic activation, resulted in detachment of most of the ribosomes. The resulting membranes resembled those seen after exposure to inorganic pyrophosphate.

5. The enzymatic activity of smooth microsomal membrane subfractions was capable of a percentage increase *in vitro* as great or greater than that of ribosome-rich rough membranes.

6. Activation *in vitro* of these membrane enzymes is probably due to conformational changes in the membrane itself rather than to removal of ribosomes from their membrane attachment.

INTRODUCTION

Glucose-6-phosphatase (EC 3.1.3.9) and such related enzymatic activities as microsomal pyrophosphatase, inorganic pyrophosphate–glucose phosphotransferase, inorganic pyrophosphate–glycerol phosphotransferase, and nucleoside di- and tri-

phosphatases, have been shown to occur attached to or as integral parts of the structure of the membranes of the endoplasmic reticulum of liver, kidney and intestinal mucosal cells[1-5]. These enzymatic activities are susceptible to great quantitative variation *in vivo* in response to such stimuli as fasting and differing levels of insulin and adrenal cortical steroids[6-8]. In addition, the apparent activity levels observed for these enzymes have been found to vary greatly with the conditions of the assays and the pretreatment of the endoplasmic reticular fractions assayed. Pretreatment of microsomes with suitable concentrations of such detergents as deoxycholate or Triton X-100 increase these enzymatic activities 30–300% (refs. 2,9–11). Such activation renders the enzymes very heat labile in the absence of substrate[9]. Pretreatment of microsomes with OH^-, such as NH_4OH (pH 9.5–9.8), causes a similar elevation *in vitro* in the activity of these membrane enzymes without increasing the lability of the enzymes to as great an extent[12,13].

A number of possible hypotheses may be proposed to explain these *in vitro* activations. The enzymes might exist in different allosteric forms or in activated or unactivated conditions with differing kinetic properties. The apparent K_m of glucose-6-phosphatase is lowered by treatment with detergents or NH_4OH (refs. 11 and 12), but the significance of K_m values obtained for particulate-bound enzymes is certainly questionable. Alternatively the activation could be the result of conformational or solubility changes in the membrane structure itself, resulting in greater accessibility of active groups to substrate molecules. One such change, as has been suggested[13], might be removal of ribosomal particles from microsomal membranes.

The present study was undertaken to explore this possibility and to compare the electron microscopic appearance of variously treated microsomal fractions with the corresponding patterns of enzyme activation. Whole untreated microsomes and their rough and smooth subfractions have been compared with fractions pretreated with NH_4OH, deoxycholate or inorganic pyrophosphate. Four enzymatic activities, glucose-6-phosphatase, inorganic pyrophosphatase, inorganic pyrophosphate–glucose phosphotransferase and inorganic pyrophosphate–glycerol phosphotransferase have been measured.

METHODS

Except where otherwise indicated, the methods of preparation of microsomal fractions of liver and the enzymatic assays were carried out as previously described[2,4,13]. Adult male rats of the Wistar strain, fasted overnight and weighing about 200–300 g, were used. Homogenization was performed in isotonic sucrose in the absence of EDTA. Microsomal fractions resuspended in isotonic sucrose (2 ml per g liver) were assayed either fresh or after freezer storage. Details of the pretreatment procedures are given in the legends to individual tables and figures.

Aliquots of resuspended and pretreated microsomes were centrifuged for 1 h at 105000 $\times g$ to obtain pellets for electron microscopy. The surface of the pellets was rinsed with isotonic sucrose. Thus the time at which fixation began was about 2 h after the final pretreatment. In the initial experiments, samples were taken by microspatula from the top, middle and the small, dark, disc-shaped bottom of each pellet. Although the latter was contaminated with mitochondria, it was a small part of the pellet. The top and middle portions of the pellet were free of contamination and

virtually indistinguishable in the electron microscope. In the later experiments, only the top portion of each pellet was sampled. Fixation was in 1% OsO_4 (ref. 17) in veronal–acetate buffer (pH 7.4) at 0° for 1 h. The pellet samples remained cohesive through the dehydration steps. Embedding was in Epon 812 (ref. 18). Sections were doubly stained with uranyl acetate[19] and lead citrate[20] and examined in the RCA EMU-3F and Philips 300 electron microscopes.

Rough and smooth subfractions of liver microsomes were prepared by the method of DALLNER *et al.*[15]. RNA determinations were by the method of MUNRO AND FLECK[16].

RESULTS

Activation of microsomal enzymes by NH_4OH and deoxycholate as a function of time at 0° and 30°

The results of a typical activation experiment are recorded in Fig. 1. The levels of each of the three enzyme activities in the control microsomes remained approximately constant at 0° for 22 h. Treatment with 0.2% deoxycholate produced the expected immediate activation at 0°. Loss of activity in deoxycholate-treated microsomes was slow at 0° and very rapid at 30°. The enzyme activities were much

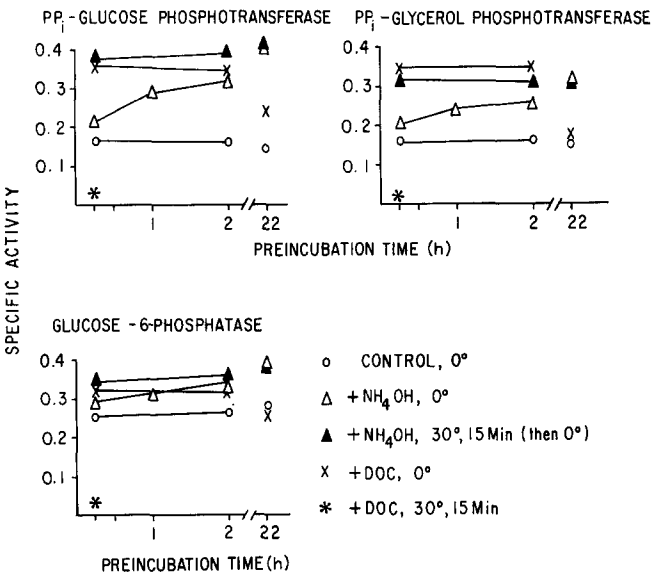

Fig. 1. Activation of microsomal enzymes by NH_4OH or deoxycholate (DOC) as a function of time at 0 and 30°. Results of a typical experiment. Microsomes of fasted rat liver were obtained by differential centrifugation of homogenates prepared in isotonic sucrose. Before assay, portions of unfrozen, freshly prepared microsomes were treated at 0° to produce a final concentration of 0.1 M NH_4OH and a pH of about 9.8, or a final concentration of 0.2% deoxycholate at pH 7.0 or 0.25 M sucrose at pH 7.0 for control determinations. After standing at 0 or 30° for different time intervals, aliquots of the enzyme samples were assayed by incubation at 30° for 10 min with optimum concentrations of substrate. The glucose-6-phosphatase assays were done at pH 6.0 in cacodylate buffer[13]. Inorganic pyrophosphate–glucose and –glycerol phosphotransferase assays were carried out at pH 5.4 by the radioactive pyrophosphate method[4,14]. Enzymatic specific activity units are μmoles of glucose 6-phosphate hydrolyzed or glucose 6-phosphate or glycerol 1-phosphate synthesized per min per mg of protein.

more stable when induced by NH_4OH than by deoxycholate[12]. When fresh unfrozen microsomes were treated with 0.1 M NH_4OH and maintained at 0°, activation was not instantaneous. The enzymatic levels rose gradually over a period of several hours. A 15- or 30-min treatment at 30° with NH_4OH produced approximately quantitatively comparable enzymatic activation to that produced by NH_4OH at 0° in 22 h or by deoxycholate instantaneously at 0°. A similar 15-min warming of control untreated microsome samples caused no activation.

With microsomes stored frozen and treated with NH_4OH after being thawed, approximately the same rate of activation and maximum enzyme levels were obtained as with fresh preparations. In earlier studies[12,13], nearly maximum enzymatic activity was observed immediately after thawing because NH_4OH had been added before the microsomal preparations were frozen.

Electron micrographic studies of pellets prepared from activated and unactivated microsomes

Freshly prepared unfrozen microsomes were treated and assayed as described in the caption to Fig. 1. To prepare pellets for electron microscopic examination, aliquots were centrifuged at about 0° at 105000 × *g* for 1 h. Approx. 2 h elapsed between the pretreatment with NH_4OH or deoxycholate and the fixation of portions of the pellet with OsO4. Typical electron micrographs of a number of the samples are shown in Fig. 2, with the corresponding percentage activations given in Table I.

Fig. 2 A is a normal control sample showing ribosomes attached to the rough endoplasmic reticular membranes. Essentially the same picture was obtained from samples kept at 0° overnight or warmed at 30° for 15 min or frozen immediately after isolation and studied after being thawed at a later time. These procedures caused neither activation of the enzymes nor appreciable removal of ribosomes. After treatment with 0.2 % deoxycholate at 0°, resulting in optimal instantaneous activation

TABLE I

ACTIVATION OF MICROSOMAL MEMBRANE ENZYMES BY NH_4OH OR DEOXYCHOLATE CORRESPONDING TO ELECTRON MICROGRAPHS OF FIG. 2

Freshly prepared unfrozen microsomes were obtained and assayed as in Fig. 1. Enzyme treatment includes time of pretreatment *plus* time of centrifugation prior to OsO_4 fixation of pellet. Results are expressed in percentage activation $\left(\frac{\text{activity of pretreated microsomes}}{\text{activity of control microsomes}} - 1\right) \times 100$.

Conditions	*Electron micrograph Fig. 2*	*PP_i–glucose phosphotransferase*	*PP_i–glycerol phosphotransferase*	*Glucose-6-phosphatase*
Control in 0.25 M sucrose				
0°, 2 h	A	0	0	0
0°, 22 h	≡A	− 16	0	+ 8
30°, 15 min + 0°, 2 h	≡A	− 15	− 3	− 6
30°, 15 min + 0°, 22 h + Deoxycholate (0.2 %)		− 15	− 5	+ 7
0°, 2 h + NH_4OH (0.1 M)	E	+102	+122	+26
0°, 2 h	B	+ 78	+ 57	+24
0°, 22 h	D	+136	+ 98	+48
30°, 15 min + 0°, 2 h	C	+130	+ 91	+37

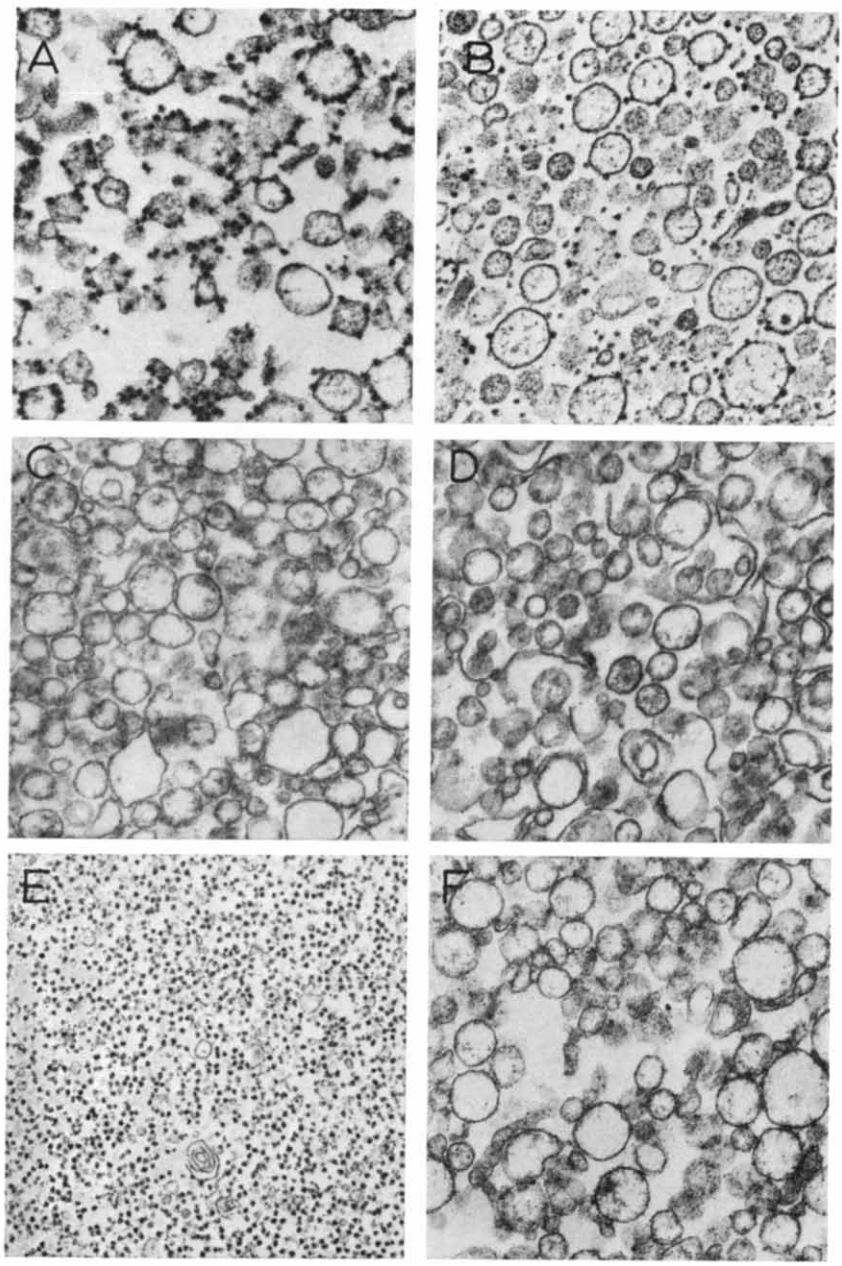

Fig. 2. Electron micrographs of sections of rat-liver microsome pellets (× 40000). About 2 h at 0° elapsed between pretreatment of the microsomes and the start of fixation of pellets with OsO_4 in all cases. A. Control microsome samples prepared in 0.25 M sucrose at 0°. Essentially identical electron micrographs obtained with samples warmed 15 min at 30°, or kept at 0° for 22 h or stored frozen and then thawed. B. Microsomes pretreated at 0° with 0.1 M NH_4OH (pH 9.8). C. Microsomes similarly pretreated with NH_4OH and then warmed for 15 min at 30°. D. Microsomes pretreated with NH_4OH and kept for 22 h at 0°. E. Microsomes pretreated at 0° with 0.2% deoxycholate (pH 7.0). F. Microsomes pretreated at 0° with 0.05 M inorganic pyrophosphate (pH 7.0).

of the microsomal enzymes, electron micrographs (Fig. 2E) were similar to those obtained by ERNSTER *et al.*[5]. Ribosomes appeared to be completely removed from the mebranes which were partially solubilized[5,11].

Electron micrographs of freshly prepared microsomes, treated at 0° with NH_4OH (Fig. 2B), showed that the ribosomes were still largely attached to the membranes. The appearance was qualitatively like the untreated controls while more than 50 % of the maximum stimulation of the three enzymatic activities had occurred. More vigorous treatment, such as warming at 30° for 15 min (Fig. 2C) or standing at 0° overnight (Fig. 2D) effecting maximum enzymatic activation (Table I), removed the ribosomes from the membranes, which remained in the form of spheres of about 2100 Å maximum diameter.

SACHS[21] showed that inorganic pyrophosphate, one of the substrates in several of our enzymatic reactions, removed most of the ribosomes from microsomal membranes. Microsomes treated with 0.05 M inorganic pyrophosphate (pH 7.0) (Fig. 2F) resembled those exposed for a prolonged period to NH_4OH (Fig. 2D). In both cases, the electron micrographs show projections from the outer surfaces of the microsomal membranes that suggest a retention of portions of subunits of ribosomes (Fig. 3). The appearance of the trilaminar unit membrane structure is unaffected by either inorganic pyrophosphate or NH_4OH treatment.

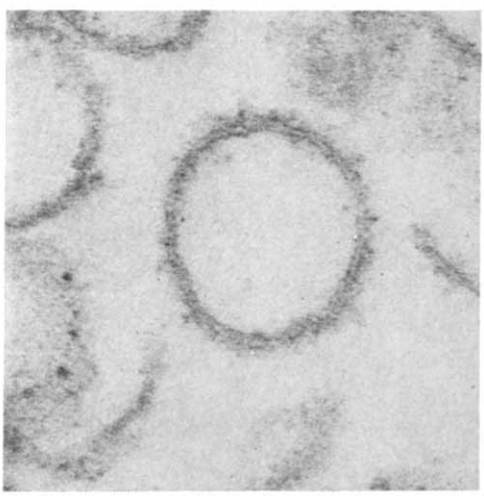

Fig. 3. Section of a microsome pretreated at 0° for 22 h with 0.1 M NH_4OH prior to fixation (× 200000).

Activation of "rough" and "smooth" fractions of endoplasmic reticulum

Microsomes were separated by centrifugation on a two-layered sucrose gradient containing CsCl (ref. 15) into sedimenting and floating subfractions, corrresponding approximately to rough and smooth microsomes. Samples were stored frozen and studied later when freshly thawed. Results of analyses and enzymatic assays of these fractions in a typical experiment are given in Table II. The total protein was approximately evenly divided between rough and smooth microsomes. The rough ones had an RNA : protein ratio about 4 times that of the smooth. Corresponding electron micrographs showed that the rough fraction consisted largely of microsomes

TABLE II

ACTIVATION *in vitro* OF ENZYMES OF LIVER ENDOPLASMIC RETICULUM AND ROUGH AND SMOOTH SUBFRACTIONS

Microsomes, prepared from livers of 24-h fasted rats, were fractionated by means of a two-layered sucrose gradient containing CsCl (ref. 15.). Fractions were stored in a concentration equivalent to 2 ml per g liver and were thawed and pretreated at 0° as indicated immediately before enzymatic assays were performed[2,4,13]. Results of a typical experiment are reported here.

Enzyme fraction	*Pretreatment*	*Protein (mg/ml)*	*Ratio RNA/protein (mg/mg)*	*Enzyme activity (μmoles substrate reacting per min per mg protein)*	
				Glucose-6-phosphatase	*Pyrophosphate–glucose phosphotransferase*
Unfractionated microsomes	None	15.2	0.12	0.255	0.164
	NH_4OH			0.307	0.338
	Deoxycholate			0.341	0.484
Rough microsomal subfraction	None	8.4	0.21	0.354	0.405
	NH_4OH			0.410	0.640
	Deoxycholate			0.416	0.510
Smooth microsomal subfraction	None	7.0	0.05	0.191	0.093
	NH_4OH			0.222	0.213
	Deoxycholate			0.258	0.319

with RNA particles still attached to the membranes while the smooth fraction was relatively free of attached ribosomes. Levels of glucose-6-phosphatase and of inorganic pyrophosphate–glucose phosphotransferase in the untreated fractions were somewhat higher in rough than in smooth microsomes. This agrees with the results of DALLNER[22] for glucose-6-phosphatase. Pretreatment with NH_4OH or deoxycholate resulted in elevation of the enzymatic activities of the smooth as well as the rough subfractions. In most cases, in fact, the percentage of activation was appreciably greater for the smooth than for the rough membranes. This aspect of the problem is being further studied.

DISCUSSION

Glucose-6-phosphatase and the related particulate enzymes of liver and kidney are found in the microsomal membrane itself and not in attached ribosomes[5]. The fact that the appreciable *in vitro* elevation of these enzymatic activities, produced by controlled treatment with deoxycholate, is accompanied by removal of ribosomes from the membranes suggests that ribosomal removal might cause or be related to the increased enzymatic reactivity. Electron micrographs of microsomes activated at 0° with NH_4OH show that ribosomal attachments are still largely intact at a time when a very appreciable amount of enzymatic activation has occurred. This finding is in accord with but certainly not conclusive proof of the hypothesis that activation is unrelated to ribosomal removal.

Further logical evidence comes from the results obtained in assays in which inorganic pyrophosphate acts as one of the enzymatic subtrates. Inorganic pyrophosphate is known to effect ribosomal removal[21]. Microsome fractions, treated with NH_4OH or deoxycholate, show greatly increased inorganic pyrophosphatase and

inorganic pyrophosphate–glucose phosphotransferase activities when compared with unactivated controls. Since the substrate itself effects removal of ribosomes in both activated and unactivated fractions, ribosomal removal cannot by itself explain the activation phenomenon.

More conclusive evidence comes from the finding that, after prior separation of microsomes into fractions consisting of predominantly rough or smooth membranes, both fractions are capable of activation, the enzymes of the smooth being capable of greater activation than those of the rough membranes.

Enzymatic activation *in vitro* is probably related to conformational or solubility changes of the membrane itself rather than to ribosome removal.

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