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DIFFERENT PROPERTIES OF GLUCOSE-6-PHOSPHATASE AND RELATED ENZYMES IN ROUGH AND SMOOTH ENDOPLASMIC RETICULAR MEMBRANES

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SUMMARY

1. Evidence presented supports the hypothesis that the endoplasmic reticular membrane enzyme, glucose-6-phosphatase, with its related activities, inorganic pyrophosphatase and inorganic pyrophosphate-glucose phosphotransferase, exists within the membrane in two different forms or different degrees of accessibility to substrate. In membrane areas having attached ribosomes the enzyme is predominately in an "activated" configuration while that in the smooth membranes is in a less active or potential form.

2. The three enzyme activities studied have higher pH optima and, under normal assay conditions, greater specific activities in the ribosome-rich, "rough" subfractions of liver microsomes than in "smooth" subfractions of the same preparations.

3. On activation by pretreatment with NH_4OH or deoxycholate, the enzymatic activities and pH optima of the smooth subfractions are shifted to higher values to a greater extent than are those of the rough.

4. When comparisons are made on optimally activated samples, total, rough and smooth membranes exhibit approximately the same enzymatic activities and identical elevated pH optima.

5. Despite great quantitative differences in the enzymatic activities of preparations from livers of fed, fasted, phenobarbital-treated and alloxan-diabetic rats, the same patterns of differences between enzyme activities of rough and smooth membranes was observed for all animals.

6. Phenobarbital-treated animals, in which there is a large proliferation of smooth membranes, have been found by others to exhibit a lower specific activity of liver glucose-6-phosphatase than do control animals. This may be explained in part by the preponderance of less active enzyme in the smooth membranes.

INTRODUCTION

The liver and kidney microsomal membrane enzyme, glucose-6-phosphatase, and such related enzymatic activities as microsomal inorganic pyrophosphatase and inorganic pyrophosphate-glucose (or glycerol) phosphotransferases are known to be subject to an appreciable activation *in vitro* by suitable pretreatment with such re-

agents as deoxycholate[1] or NH_4OH (ref. 2). In the course of a study of the relationship between microsomal membrane structure and this enzymatic activation we found that smooth microsomal membrane fractions were capable of a greater degree of activation than were the ribosome-rich rough membranes[3]. The present study extends this observation and explores the possibility that the results may explain several observed properties of glucose-6-phosphatase.

METHODS

Male rats of the Wistar strain, fed, fasted and alloxan diabetic, weighing between 200 and 300 g, were prepared as previously described[4,5]. Phenobarbital-treated animals received intraperitoneal injections of 80 mg of sodium phenobarbital per kg of rat per day for five days[6].

In most of the experiments a 1:4 homogenate of liver was prepared in 0.25 M sucrose and cellular debris and mitochondria were removed by centrifugation at 10000 × *g* for 20 min. Rough and smooth microsomal subfractions were prepared by differential centrifugation with a two-layered sucrose gradient containing CsCl by the method of DALLNER *et al.*[7,8]. A clear separation of the two fractions was obtained by centrifuging for 210 min at 39000 rev./min in a SW 39 rotor of a Spinco Model L centrifuge[8]. Portions of total microsomes in 0.25 M sucrose, containing 15 mM CsCl, and of rough and smooth microsomal subfractions, resuspended in 0.25 M sucrose, were either analyzed at once or frozen for several days before use. In a few experiments microsomal subfractions were prepared by centrifugation of diluted mitochondrial supernatant fractions on a discontinuous sucrose gradient for 8 h in a No. 40 rotor by the method of ROTHSCHILD[9].

Enzymatic assays, protein determinations, and pH–activity curves were carried out as previously described[3,5,10]. Activation of the enzyme preparations with NH_4OH or deoxycholate and stability studies were done by methods indicated for each experiment. Ribonucleic acid was estimated by the ultraviolet absorption method, as modified for rat liver by MUNRO AND FLECK[11].

RESULTS

Enzyme activities of rough and smooth subfractions of liver microsomes before and after maximum activation with NH_4OH

Electron micrographs, prepared by Dr. Sasha Malamed of the Anatomy Department of Rutgers Medical School, of subfractions obtained by differential centrifugation showed that the microsomes had been separated into ribosome-rich "rough", and essentially ribosome-free "smooth" fractions, whose appearance was similar to that observed by DALLNER *et al.*[7,8].

The specific activities of inorganic pyrophosphate-glucose phosphotransferase, determined on freshly thawed samples of total, rough and smooth liver microsomes are given in Table I. In agreement with earlier results[4] the enzyme activities of total microsomes were found to be greatly elevated in fasted and in diabetic animals when compared with normal fed controls. Enzyme levels in untreated microsomes were lower in animals given phenobarbital than in normal animals, in confirmation of the work of others[22,23]. In all groups studied, in spite of the great quantitative difference

TABLE I

INORGANIC PYROPHOSPHATE-GLUCOSE PHOSPHOTRANSFERASE ACTIVITY OF LIVER MICROSOMES AND THEIR ROUGH AND SMOOTH SUBFRACTIONS

Enzyme samples analyzed were: 1. total liver microsomes. 2. rough, ribosome-rich, and 3. smooth, essentially ribosome-free, microsomal subfractions prepared as described in METHODS. Each sample was resuspended in 0.25 M sucrose at a concentration of about 10 mg protein per ml and stored frozen for several days. Samples, freshly thawed at 0°, were treated with either 1 volume of isotonic sucrose or 1 volume of 1 M NH_4OH per 10 volumes of enzyme suspension for analysis as "untreated" or "NH_4OH-treated" microsomes respectively. NH_4OH-treated samples were kept at 30° and analyzed at different time intervals to obtain maximum values. Enzymatic assay mixtures consisted of 0.08 M inorganic pyrophosphate and 0.4 M glucose in acetate buffer at pH 5.2. Reaction was started by the addition of a small volume of enzyme at 0° to the substrate at 30°, the final mixture containing about 1 mg protein per ml. After incubation at 30° for 10 min the reaction was stopped by heating for 3 min at 100° and the amount of glucose-6-phosphate formed was measured. Specific activities are given as mean values, followed by S.E. Differences observed between untreated rough and smooth microsomes are in all cases statistically significant ($P<0.01$) whereas the smaller differences observed after activation with NH_4OH are not statistically significant.

Rats condition	*Microsome fraction*	*Microsomal*		*Enzyme activity (μmoles glucose-6-P formed/min per mg protein)*		*Maximum activation* (%)
		Protein (mg/g liver)	*RNA*	*Untreated*	*NH_4OH-treated*	
Normal, fed	Total	34.7	2.2	0.123 ± 0.009	0.255 ± 0.016	108 ± 5
(3)	Rough	13.4	1.7	0.183 ± 0.015	0.227 ± 0.017	24 ± 7
	Smooth	14.7	0.6	0.110 ± 0.002	0.260 ± 0.010	137 ± 14
Normal, fasted	Total	42.3	4.3	0.218 ± 0.013	0.460 ± 0.033	111 ± 7
(5)	Rough	15.9	2.9	0.329 ± 0.018	0.499 ± 0.053	50 ± 8
	Smooth	17.4	0.8	0.199 ± 0.015	0.430 ± 0.046	114 ± 10
Phenobarbital, fed	Total	46.0	2.4	0.101 ± 0.007	0.257 ± 0.007	156 ± 11
(3)	Rough	17.3	1.7	0.155 ± 0.012	0.178 ± 0.012	15 ± 3
	Smooth	19.0	0.6	0.088 ± 0.004	0.193 ± 0.004	121 ± 13
Phenobarbital, fasted	Total	59.3	3.3	0.127 ± 0.007	0.346 ± 0.007	173 ± 20
(3)	Rough	16.3	2.0	0.256 ± 0.016	0.321 ± 0.016	16 ± 1
	Smooth	30.9	0.8	0.124 ± 0.005	0.315 ± 0.005	154 ± 26
Diabetic, fed	Total	36.6	2.5	0.310 ± 0.052	1.025 ± 0.052	243 ± 53
(3)	Rough	15.1	1.8	0.484 ± 0.067	0.846 ± 0.067	84 ± 23
	Smooth	14.8	0.4	0.304 ± 0.033	1.054 ± 0.033	250 ± 21

in the absolute levels of the enzyme between groups of animals, the enzyme activity of the rough microsomes measured at pH 5.2 was highly significantly greater ($P<0.01$) than that of the smooth when untreated microsomes were studied. In contrast, when measured in the same way after optimal activation by pretreatment with NH_4OH, the enzyme levels of rough and smooth microsomes did not differ significantly from each other. In fact, if the somewhat greater instability of the enzyme in the rough membranes, which will be discussed later, is taken into account, it appears that the potential available enzyme activity is about the same for the two types of membranes.

Some insight into the nature of the differences observed with untreated microsomes can be obtained by comparing the results when expressed as maximum percentage of activation (last column, Table I). The enzyme activity of the smooth membrane fraction is seen to be greatly increased by pretreatment with NH_4OH.

The degree of activation observed varied from more than 100 % in normal to 250 % in the case of diabetic animals. The rough microsomal fractions, in contrast, were capable of much less activation by a similar pretreatment. Thus the initially higher enzyme level of the rough membranes is due to the fact that these preparations are already in a condition or configuration favorable for reaction with substrate molecules under the conditions used for the assays, while the smooth membranes are in a more inhibited state.

In Table II are given the results of glucose-6-phosphatase assays, carried out at the same time as the inorganic pyrophosphate-glucose phosphotransferase assays and on the same liver microsomes and their rough and smooth subfractions. With a few exceptions the results obtained with glucose-6-phosphatase and inorganic pyrophosphatase were parallel to those with the transferase activity, in accord with the evidence of the probable identity of these enzymatic activities[4,12,13]. Except for the diabetic animals, to be discussed later in connection with the observed pH optimum shift, the untreated rough microsomes showed somewhat higher glucose-6-phosphatase activities than did the corresponding smooth microsome fractions. Although there was no overlap of absolute values obtained with different individual animals between rough and smooth untreated microsomes, the average differences were not statistically significant ($P>0.05$) in these series. We have consistently observed[10,5] that the glucose-6-phosphatase activity is not capable of as great a degree of *in vitro* activation

TABLE II

GLUCOSE-6-PHOSPHATASE ACTIVITY OF LIVER MICROSOMES AND THEIR ROUGH AND SMOOTH SUBFRACTIONS

Enzyme preparation and preincubation and assay conditions were the same as those described in Table I. Assay mixtures contained 0.08 M glucose-6-phosphate in 0.08 M cacodylate buffer at pH 6.0. After 10 min incubation at 30°, enzymatic reactions were stopped by the addition of trichloroacetic acid and the inorganic phosphate formed was measured. Mean values are given followed by the S.E.

Rats condition	*Microsome fraction*	*Enzyme activity (μmoles P_i formed/min per mg protein)*		*Maximum activation (%)*
		Untreated	*NH_4OH-treated*	
Normal, fed (3)	Total	0.147 ± 0.007	0.178 ± 0.010	22 ± 1
	Rough	0.160 ± 0.008	0.167 ± 0.011	4 ± 3
	Smooth	0.147 ± 0.004	0.183 ± 0.002	25 ± 2
Normal, fasted (5)	Total	0.217 ± 0.010	0.291 ± 0.015	34 ± 2
	Rough	0.256 ± 0.013	0.324 ± 0.020	26 ± 3
	Smooth	0.206 ± 0.016	0.273 ± 0.019	33 ± 4
Phenobarbital, fed (3)	Total	0.117 ± 0.010	0.158 ± 0.009	36 ± 5
	Rough	0.133 ± 0.014	0.145 ± 0.013	10 ± 2
	Smooth	0.105 ± 0.003	0.125 ± 0.003	19 ± 6
Phenobarbital, fasted (3)	Total	0.150 ± 0.003	0.218 ± 0.008	45 ± 3
	Rough	0.176 ± 0.012	0.205 ± 0.008	17 ± 4
	Smooth	0.143 ± 0.004	0.204 ± 0.018	42 ± 13
Diabetic, fed (3)	Total	0.352 ± 0.022	0.657 ± 0.063	77 ± 6
	Rough	0.399 ± 0.021	0.559 ± 0.025	40 ± 3
	Smooth	0.432 ± 0.020	0.720 ± 0.063	66 ± 7

as is the pyrophosphate-glucose phosphotransferase activity of liver microsomes. The present results with fractionated as well as total microsomes are in agreement with this finding. When the results are expressed as maximum percentage of activation produced by NH_4OH pretreatment, the glucose-6-phosphatase of the smooth membranes is seen in all cases to be activated to a significantly greater extent than is that of the rough membranes.

The corresponding data for the microsomal inorganic pyrophosphatase assays are given in Table III. Enzyme levels measured on the untreated, rough membranes were in all cases significantly higher ($P<0.01$) than those of the untreated smooth membranes, while pretreatment with NH_4OH produced differential activation which tended to eliminate the differences between the membranes with and without attached ribosomes. The maximum activation effect for inorganic pyrophosphatase was quantitatively intermediate between that of glucose-6-phosphatase and inorganic pyrophosphate-glucose phosphotransferase, as noted earlier for total microsomes[10]. The much greater potential for activation of the enzyme of the smooth than of the rough membranes can be seen to be a reflection of the initially higher levels of active enzyme in the rough subfraction, as was the case for the other activities measured.

Control experiments. In considering whether or not the observed differences between the properties of the membrane enzymes of rough and smooth microsomal subfractions were due to preexisting differences in the membranes, it seemed necessary to consider and to eliminate, in so far as feasible, the possibility that the differences were induced *in vitro* by the isolation or storage procedures used.

TABLE III

INORGANIC PYROPHOSPHATASE ACTIVITY OF LIVER MICROSOMES AND THEIR ROUGH AND SMOOTH SUBFRACTIONS

Enzyme preparations and preincubation and assay conditions were the same as those described in Table I. Assay mixtures contained 0.08 M inorganic pyrophosphate in acetate buffer at pH 5.4.

Rats condition	*Microsome fraction*	*Enzyme activity (μmoles substrate used/min per mg protein)*		*Maximum activation* (%)
		Untreated	*NH_4OH-treated*	
Normal, fed (3)	Total	0.106 ± 0.008	0.198 ± 0.021	87 ± 15
	Rough	0.148 ± 0.007	0.169 ± 0.010	14 ± 1
	Smooth	0.092 ± 0.001	0.193 ± 0.004	109 ± 4
Normal, fasted (5)	Total	0.168 ± 0.003	0.262 ± 0.005	56 ± 2
	Rough	0.233 ± 0.005	0.272 ± 0.021	17 ± 7
	Smooth	0.170 ± 0.003	0.268 ± 0.013	58 ± 13
Phenobarbital, fed (3)	Total	0.085 ± 0.015	0.173 ± 0.014	112 ± 21
	Rough	0.134 ± 0.027	0.158 ± 0.029	17 ± 3
	Smooth	0.072 ± 0.014	0.137 ± 0.009	101 ± 30
Phenobarbital, fasted (3)	Total	0.104 ± 0.003	0.218 ± 0.003	111 ± 2
	Rough	0.202 ± 0.026	0.230 ± 0.038	14 ± 4
	Smooth	0.100 ± 0.003	0.208 ± 0.023	110 ± 28
Diabetic, fed (3)	Total	0.312 ± 0.049	0.803 ± 0.104	160 ± 17
	Rough	0.450 ± 0.061	0.653 ± 0.044	48 ± 10
	Smooth	0.294 ± 0.040	0.838 ± 0.085	188 ± 9

TABLE IV

CONTROL EXPERIMENTS

A pellet of unfractionated (total) microsomes from the liver of a fasted rat was divided into two portions. One portion was suspended in hypertonic (1.3 M), the other in isotonic (0.25 M) sucrose. Rough and smooth microsomal subfraction were prepared from the same liver homogenate by the method of DALLNER *et al.*[8]. The smooth membrane fraction, accumulated at the phase interface, was collected, divided into two portions, and one portion adjusted to about 1.3 M and the other to 0.25 M sucrose. After all samples had been kept at 0° for 3.5 h, the sucrose concentrations were adjusted to 0.25 M, the particulate fractions centrifuged, resuspended in isotonic sucrose and stored frozen before enzymatic assay. In the study of the possible effect of CsCl upon the enzyme activity, microsomes were isolated from the liver of a normal fed rat by two methods: in isotonic sucrose with and without 15 mM CsCl. Enzymatic analyses were carried out both on freshly prepared unfrozen microsomes and on portions stored frozen and thawed just before assay. Activation was carried out by pretreatment of the microsomes in 0.1 M NH_4OH for 15 min at 30°.

Microsomes	*Enzyme activity (μmoles substrate used/min per mg protein)*		
	PP_i-glucose phospho-transferase	*Glucose-6-phosphatase*	*Inorganic pyrophosphatase*
Total — 0.25 M sucrose, not activated	0.187	0.178	0.164
1.3 M sucrose, not activated	0.193	0.182	0.155
Rough — 1.3 M sucrose, not activated	0.277	0.210	0.224
Smooth — 0.25 M sucrose, not activated	0.140	0.112	0.123
1.3 M sucrose, not activated	0.149	0.129	0.128
Total — no CsCl, unfrozen, not activated	0.109	0.133	
no CsCl, frozen, not activated	0.110	0.140	0.096
with CsCl, unfrozen, not activated	0.108	0.134	
with CsCl, frozen, not activated	0.105	0.137	0.092
Total — no CsCl, unfrozen, activated	0.230	0.172	
no CsCl, frozen, activated	0.224	0.168	0.172
with CsCl, unfrozen, activated	0.220	0.162	
with CsCl, frozen, activated	0.227	0.163	0.158

In the method used for separating rough from smooth membranes, total microsomal preparations in 0.25 M sucrose were layered over 1.3 M sucrose, both solutions containing CsCl. The rough microsomes, collecting at the bottom of the tubes during a 3.5-h centrifugation, were thus, for an appreciable time, in contact with a more hypertonic sucrose solution than were the smooth microsomes which accumulated at the interface between the phases. The results of an experiment, in which total and smooth microsomes were exposed to a hypertonic solution of sucrose to an extent comparable with the necessary exposure of the rough microsomes during the fractionation procedure, are given in the upper portion of Table IV. It can be seen, for all three enzyme activities measured, that exposure of total or of smooth membranes to a hypertonic sucrose solution does not cause significant activation. Thus it appears likely that the higher enzyme activity observed for the rough membranes is due to a preexisting property of the membrane and not produced by exposure to a more hypertonic solution during the fractionation procedure.

The sedimentation method used for separation of microsomes has been shown[14] to depend upon a selective aggregation of the particles in the presence of CsCl. The data of the lower portion of Table IV shows that the presence or absence of CsCl has

no significant effect upon the enzyme levels found with untreated or NH_4OH-activated microsomes, whether analyzed fresh or after having been stored frozen.

Results with other methods of fractionation or activation. When microsomal subfractions are prepared without the aid of CsCl or other monovalent cations, a longer period of centrifugation is required to obtain adequate separation. In a few experiments rough and smooth subfractions, prepared by an 8-h centrifugation of liver microsomes on a two-layered sucrose gradient as described by ROTHSCHILD[9] were analyzed for inorganic pyrophosphate-glucose phosphotransferase and glucose-6-phosphatase both before and after NH_4OH pretreatment. The results were approximately the same as those obtained with samples prepared by the method of DALLNER[7] except that the membrane enzymes, particularly in the rough fractions, were appreciably more unstable. The initial measurable enzyme activities were higher in the rough than in the smooth fractions and the smooth were always capable of a much greater increase in activity by pretreatment with NH_4OH. The increased instability of these preparations, reflected in some cases in an absolute decrease in activity of rough membranes on NH_4OH treatment, made the quantitative results less useful than those obtained with CsCl.

When suitable concentrations of deoxycholate or of Triton-X-100 were used instead of NH_4OH to activate the membrane enzymes, the results resembled those obtained after NH_4OH activation. The enzymes of smooth membranes, initially more inhibited, were capable, under optimal conditions, of a greater percentage of increase in activity than were those of rough membranes when treated with detergent either prior to or during the enzymatic assay. However, the effect of detergents on glucose-6-phosphatase and related enzyme activities, whether they produce preponderantly activation, inhibition or destruction, is critically dependent, in an unpredictable way, upon the nature, the concentration and the manner of application of the particular detergent used[1,10,15,16]. In addition, the detergents render the membrane enzymes extremely susceptible to heat denaturation[15,10]. We have found it possible to obtain more quantitatively consistent results, in these as in earlier studies[3,10], by using NH_4OH rather than detergents as the activating reagent.

Thus, the observed differences in initial activity and in susceptibility to further activation between rough and smooth microsomal fractions is not due to the use of a specific method of fractionating or of producing maximum activation.

Stability of enzymes in presence of substrate

The enzyme activities of the rough and smooth subfractions as well as of the total microsome fractions were found to be stable in the presence of substrate under the various assay conditions used in these studies. With high substrate concentrations, glucose-6-phosphatase and the other enzyme activities proceeded at an approximately constant rate with time for periods of at least 30 min at 30°. This proved to be true for total, rough and smooth microsomal fractions, both untreated and NH_4OH- or detergent-pretreated preparations and for reactions run at a variety of pH levels. There appeared to be no appreciable enzyme activation or inactivation under the conditions of the 10-min assays used.

Enzyme activation and stability in the absence of substrate

Under optimal conditions of isolation and storage the membrane enzymes of

liver microsomes are relatively stable at 30°. A typical example is given in Fig. 1. Total microsomes and their smooth subfractions, analyzed without pretreatment either immediately after isolation or after having been stored frozen for a few days or weeks, could be preincubated for at least 2 h without appreciable loss of activity (Fig. 1, open circles). Under the same conditions rough subfractions lost activity at a slow rate.

We have previously shown, using total microsome fractions, that enzymatic activation by NH_4OH (pH 9.5–10.0) is not instantaneous[3]. 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°. In the present experiments (Fig. 1, solid circles), it was shown that the rate of attainment of maximum enzyme activity by pretreatment with NH_4OH at 30° was much greater for rough than for smooth microsomes. In most cases the enzymes of the rough fractions were fully activated within 15 min while 30 min to 1 h were required for those of smooth fractions. Activated rough membranes lost activity

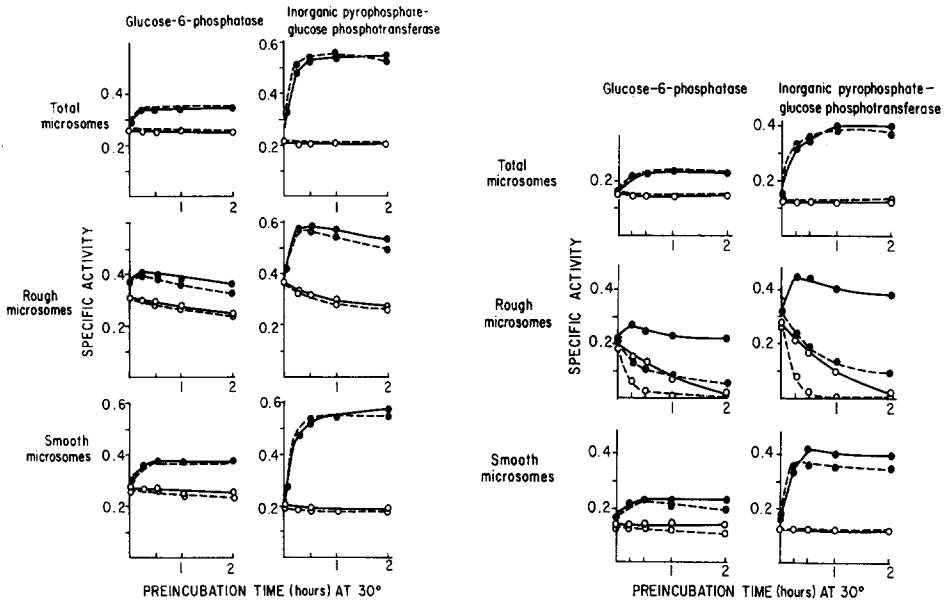

Fig. 1. Enzyme activation and stability at 30° in the absence of substrate. Typical example obtained under optimal conditions. Freshly prepared, unfrozen rat (24 h fasted) liver microsomes and their rough and smooth subfractions[8] were preincubated at 30° both with and without pretreatment with NH_4OH as described in Table I. Aliquots of enzyme were taken at different times during the preincubation period and assayed for glucose-6-phosphatase in cacodylate buffer at pH 6.0 and for inorganic pyrophosphate-glucose phosphotransferase in acetate buffer at pH 5.2. Parallel experiments were carried out using freshly thawed portions of the same enzyme samples which had been stored frozen for several days. Coinciding points are indicated by one point. ———, fresh microsomes; – – –, frozen and thawed; ○—○, unactivated; ●—●, NH_4OH-pretreated.

Fig. 2. Enzyme activation and stability at 30° in the absence of substrate. Conditions of isolation, pretreatment and assay were, in so far as possible, identical with those of Fig. 1. This is an example of the type of instability sometimes inexplicably observed, especially for rough microsomes. Coinciding points are indicated by one point. ———, fresh microsomes; – – –, frozen and thawed; ○—○, unactivated; ●—●, NH_4OH-pretreated.

at 30° more rapidly than did their smooth counterparts. The absolute enzyme levels measured in each case were surely the resultant of the simultaneously occurring activation and inactivation processes.

In accord with the often noted instability of glucose-6-phosphatase and the general failure of all attempts to purify and isolate this phospholipid-dependent enzyme, we have often obtained preparations which exhibited greater instability than those illustrated by Fig. 1. Individual experiments varied greatly, but in general it may be stated that instability was increased by prolongation of the time or slight elevation of the temperature during centrifugation or processing of the microsomal subfractions as well as by prolonged storage in a frozen state. Fig. 2 gives an example of a preparation in which, typically, the enzymes of the rough subfraction were appreciably more labile at 30°, and especially so after having been frozen, than were those of the smooth subfractions. In such preparations the enzymes of the rough subfraction were sometimes at their highest measurable activity immediately after the addition of NH_4OH at 0°. Thus, for the data of Tables I–III, maximum values obtained by assaying at different time intervals for individual cases were used, usually time 0 or 15 min for rough and 30 or 60 min at 30° after NH_4OH addition for smooth or total microsomes.

Comparison of pH optima of enzymes of rough and smooth microsomal subfractions

We have shown that activation of total liver microsomal preparations by pretreatment with NH_4OH, deoxycholate or Triton-X-100 results in a shift in the optimum pH of membrane enzymes in the alkaline direction[10]. This shift, under our assay conditions, is from about pH 4.8 to 5.2–5.4 for PP_i-glucose phosphotransferase, from about pH 5.0 to 5.4–5.8 for inorganic pyrophosphatase, and from about 5.5 to 6.0–6.5 for glucose-6-phosphatase. If, as indicated by our comparative assays and control experiments with rough and smooth subfractions, the enzymes of the untreated rough membranes are in a relatively more activated or accessible state within the membrane, the difference might be expected to be reflected in different pH optima of the enzymes of the fractions. This has consistently been found to be true.

For example (Fig. 3a) the pH optimum for the PP_i-glucose phosphotransferase activity of the smooth membrane fraction of untreated microsomes, separated by differential centrifugation, was found to be at pH 4.8, that of the total microsomes the same or slightly higher, while that of the corresponding rough subfraction was about pH 5.1. When each of these preparations was pretreated with NH_4OH prior to assay (Fig. 3b), all three exhibited the same pH optimum, about 5.2–5.3, identical with that of variously activated microsomal preparations[10]. The separation procedure used undoubtedly results in preparations in which "rough" and "smooth" particles are not completely mutually exclusive, so even the "rough" subfraction shows a slight shift in pH optimum on activation. The results are compatible with the assumption that the "rough" fractions consist chiefly of membranes containing enzymes in a different or more activated state, having a higher pH optimum, than the enzymes of these particles which lack attached ribosomes. Pretreatment with NH_4OH converted all fractions to a state of activity and pH optimum similar to that of the untreated rough fraction.

This effect is not peculiar to NH_4OH pretreatment. The initial difference in pH optima between PP_i–glucose phosphotransferase activities of untreated rough and

smooth subfractions was similarly abolished when the preparations were pretreated at 0° with 0.2% deoxycholate at pH 7.1 instead of with NH_4OH at pH 9.8. The instability of the membrane enzyme in the presence of deoxycholate was especially noticeable for rough microsomes. Regardless of the method of "activation" used, the same shift in optimum pH of the enzyme is observed.

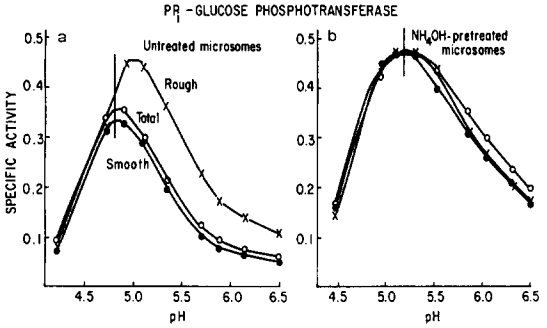

Fig. 3. pH–activity curves of inorganic pyrophosphate-glucose phosphotransferase for total, rough and smooth liver microsomes untreated (a) and pretreated with NH_4OH (b). Enzyme preparations were from a normal fasted rat. For untreated microsomes and their subfractions, small aliquots of freshly thawed samples at 0° were added to substrate–buffer mixtures of appropriate pH at 30° and the glucose-6-phosphate formed during a 10-min incubation at 30° was measured. Before assay of NH_4OH-pretreated samples, total and smooth microsomes were optimally activated by warming at 30° for 30 min after the addition of NH_4OH. Rough microsomes in this case were maintained at 0° after NH_4OH addition prior to assay. Assay reactions were stopped by heating for 3 min at 100° and the pH recorded was that measured in the reaction mixture at the end of the assay period.

For the glucose-6-phosphatase function of the membrane enzyme nearly optimum enzyme activity is obtained over a much wider range of pH values than is the case for the transferase activity. That is, flatter pH–activity curves are obtained. Nevertheless, a similar difference in pH optima can be shown. In Fig. 4, in which the ordinate scale is expanded 5-fold compared with that of Fig. 3, it can be seen that the glucose-6-phosphatase of the rough microsomal fraction has a higher pH optimum than that of the smooth fraction and that rough and smooth subfractions shift to the same higher pH optima as do total microsomes after pretreatment with NH_4OH.

The difficulties inherent in comparing absolute levels of enzyme activities of treated and untreated preparations when the pH optima change, are evident and have been discussed earlier[10]. This is also the case when activities of rough and smooth subfractions are compared and much of what is referred to as "activation" is in fact attributable to differences in pH optima. The glucose-6-phosphatase level in the case of diabetic animals was the one inconsistency noted in the general conclusion that, under the conditions used for assay, the enzyme activities in untreated preparations are higher for rough than for smooth membranes. This can be seen from Fig. 5 to be due to the pH levels selected for the assays. With untreated preparations from livers of diabetic animals there was a greater difference between pH optima for glucose-6-phosphatase of rough and smooth microsomal subfractions than was the case for the other types of animals studied. Glucose-6-phosphatase assays carried out, as in our studies recorded in Table II, at pH 6.0 or lower would make levels appear higher

in smooth than rough, while measurements made at pH 6.5, under conditions used by JAKOBSSON AND DALLNER[17] would give higher glucose-6-phosphatase levels in the rough subfractions, as found by these workers. After NH_4OH pretreatment, producing the expected elevation in enzyme activities, the pH optima of the enzyme in the total, rough and smooth subfractions from diabetic rats were identical, as with normal rats.

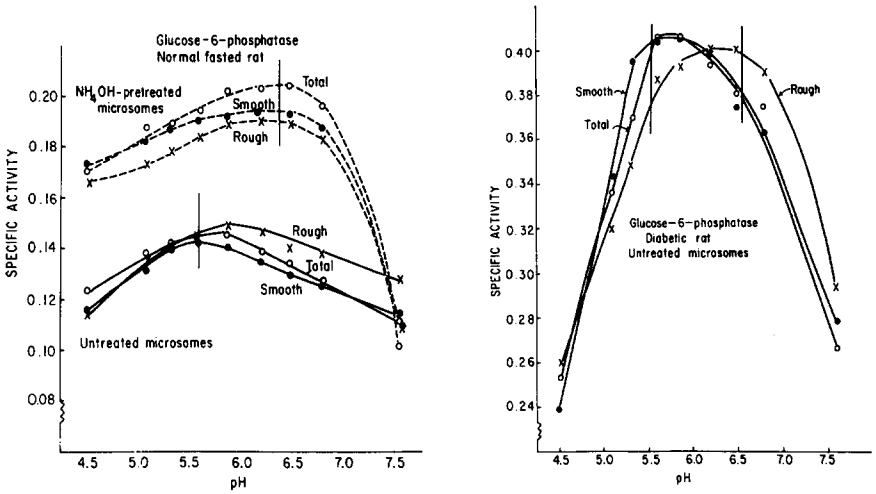

Fig. 4. pH–activity curves for glucose-6-phosphatase of total, rough and smooth liver microsomes with and without pretreatment with NH_4OH. Freshly thawed samples from a normal fasted rat were used. Preparation and pretreatment of the enzyme samples was as in Fig. 3. Glucose-6-phosphatase was determined by measuring the production of inorganic phosphate during a 10 min incubation at 30° in 0.08 M glucose-6-phosphate in mixtures of cacodylate and acetate buffers of appropriate pH. Specific activity in μmoles P_i formed/min per mg protein. Note that the ordinate is 5-fold expanded compared with Fig. 3.

Fig. 5. pH–activity curves for glucose-6-phosphatase of freshly prepared unactivated liver microsomes from an alloxan-diabetic rat. Comparable curves were obtained with frozen and thawed samples. The ordinate scale is the same as that of Fig. 4.

Michaelis constants

The Michaelis constants for the glucose-6-phosphatase activity of both rough and smooth subfractions of liver have been found to be identical with the K_m of the enzyme of the whole microsomal preparation from which the fractions were derived[17]. We have confirmed this observation and have also found no significant differences between the K_m for inorganic pyrophosphate, or for glucose, in the PP_i-glucose phosphotransferase reaction, whether determined with total, rough or smooth microsomal fractions when the state of the animal and the conditions of the assay are invariant.

DISCUSSION

Despite the recognized heterogeneity of the isolated rough and smooth subfractions of liver microsomes[18], available evidence indicates that, in the liver of adult animals, the two types of membranes, with or without attached ribosomes, usually do not differ greatly in composition, enzyme activity, or in rate of turnover of total

membrane protein or phospholipid[19,20]. The differences observed in enzyme activity levels and in rate of protein synthesis in the fractions from developing liver cells support the hypothesis that the rough are the biological precursors of the smooth membranes[8]. However, in the case of several of the enzymes which are membrane constituents, the activity levels have often been found to be significantly different in the two microsomal subfractions, even in the livers of adult animals. Glucose-6-phosphatase is one of these enzymes, the measured activity being at consistantly higher levels in the rough than in the smooth subfractions[6,7,21-23]. We have confirmed this observation for glucose-6-phosphatase and have also found the associated enzyme activities, PP_i-glucose phosphotransferase and microsomal inorganic pyrophosphatase in a variety of physiological conditions to be at a higher level in rough than in smooth membrane subfractions when measured on untreated preparations. This difference is more apparent than real, however, since, on treatment with NH_4OH or detergent in such a way as to optimally activate the enzymes, the increase in activity is chiefly in the smooth membranes and quantitative differences between the activities in rough and smooth preparations are greatly reduced. In fact, when allowance is made for the demonstrated greater lability of the enzyme in the rough fractions, it appears that the level of the total glucose-6-phosphatase may well be identical in all of these membranes.

If the conclusion is accepted that the quantity of ultimately available glucose-6-phosphatase is the same in the rough and the smooth fractions of the liver endoplasmic reticular membranes, the consistent differences initially observed in the activities measured on these untreated fractions may be due to either (1) gross configurational differences in the membranes as a whole, or (2) conformational differences in the specific protein–phospholipid enzyme itself. There has been considerable study and speculation as to the relationship of conformational or configurational structure changes to energy coupling in mitochondrial membranes[24]. A similar approach may be applicable to a study of structure relative to such functions of the endoplasmic reticular membranes as glucose-6-phosphate hydrolysis or synthesis.

Electron micrographic studies of fixed sections of liver often reveal the granular or rough surfaced endoplasmic reticulum as layers of long regularly organized, flattened cisternae while the smooth membranes present a more irregular, meshed appearance[25]. One can picture, as a simplistic explanation, that the rough membranes, perhaps stretched out by mutual repulsion of ribosomes, might permit more ready access of substrate molecules to the active sites of the enzyme areas of the membrane while, in the convoluted smooth portions of the membranes, these same sites would be less available to reactions with substrate.

Alternatively, the conformation of protein of the glucose-6-phosphatase molecule relative to the phospholipid component of the enzyme might differ depending upon the presence or absence of ribosomes on the membrane, that form existing in the rough membranes having the greater reactivity. Such a conformational difference might be expected to be reflected in a difference in the observed K_m values, which was not the case.

Administration of phenobarbital and other drugs to experimental animals has been shown to result in a spectacular increase in the quantity of smooth endoplasmic reticulum in liver cells and an accompanying increase in the activity of a number of drug-metabolizing enzymes of liver[6,26]. Resulting different levels of various individual

enzymes are thought to be due to differing alterations in the rate of synthesis or of degradation of individual proteins[27]. Glucose-6-phosphatase and a few other membrane enzymes appear to decrease in response to phenobarbital treatment[22,23]. Our results with all three of the glucose-6-phosphatase-related enzyme activities are in accord with this observation. Both in fed and in fasted animals the enzyme levels were lowered in the livers of phenobarbital treated animals. This may well be due to the effect of the accumulation of other microsomal proteins, whose degradation is retarded or whose synthesis is accelerated, on the content of an enzyme whose turnover rate is unaffected[28]. However, our results suggest a simpler explanation. The finding of a less active form of the enzyme in the smooth membranes accounts, at least in part, for the apparent overall decrease in activity in animals in which the drug has caused a specific proliferation of smooth membranes.

The extensive increase in smooth-surfaced endoplasmic reticulum noted in electron micrographs of sections of alloxan-diabetic rat liver cells[23], though not so great nor so extensively documented as in the case of phenobarbital treated animals, may be a factor in explaining the relatively greater activatability of the glucose-6-phosphatase enzymes from diabetic as compared with normal rats.

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