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SOME PROPERTIES OF VARIOUSLY ACTIVATED MICROSOMAL GLUCOSE-6-PHOSPHATASE, INORGANIC PYROPHOSPHATASE AND INORGANIC PYROPHOSPHATE–GLUCOSE PHOSPHOTRANSFERASE. SHIFT IN pH OPTIMUM

MARJORIE R. STETTEN AND FOSTER F. BURNETT
Department of Medicine, Rutgers Medical School, New Brunswick, N.J. (U.S.A.)

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SUMMARY

1. The activity of microsomal glucose-6-phosphatase (EC 3.1.3.9) and the related enzymatic functions, acid inorganic pyrophosphatase and inorganic pyrophosphate–glucose phosphotransferase is greatly increased by pretreatment of 'microsomes' with hydroxyl ion at pH 9.5–9.8. The thermal stability of the preparations so activated is in marked contrast to the great instability observed when a corresponding activation is accomplished with deoxycholate or Triton X-100.

2. It is suggested that comparative levels of these enzyme activities be determined on NH_4OH-treated homogenates or particulate fractions rather than on untreated or detergent-activated preparations.

3. After activation with NH_4OH, deoxycholic acid, or Triton X-100 the optimum pH of all three activities is shifted in the alkaline direction.

4. A similar activation and pH optimum shift is observed with liver microsomes from fed, fasted and diabetic rats, in which the absolute level of activity varies many-fold.

5. Erroneous results are obtained if activities of untreated microsomes are compared with activated ones at the same pH. This is particularly true for the transferase activity in which the quantitative effect of the pH optimum shift is much greater than for the hydrolase activities.

6. A difference between glucose-6-phosphatase and the related PP_i-utilizing enzymatic activities has been noted and studied. Glucose 6-phosphate hydrolysis by liver microsomes is inhibited by lower concentrations of deoxycholate or Triton X-100 than are required to cause activation. PP_i hydrolysis and glucose 6-phosphate synthesis from PP_i, on the other hand, are only activated by these low concentrations of detergents.

7. The results lead to the suggestion that some of the active sites, particularly those for glucose, are on the inner surfaces of the endoplasmic reticulum or are impeded by the spatial conformation of the membrane or by the attachment of ribosomes on the outer surfaces.

INTRODUCTION

In the process of homogenization of tissue the endoplasmic reticulum of the cells is pictured as being broken down by a generalized pinching-off process into the discrete vesicular and tubular fragments which constitute the 'microsomal' fraction isolated by differential centrifugation[1]. It has been shown that those enzymes of the rat-liver microsomes which are soluble, presumably those present *in vivo* loosely bound or in the lumina of the endoplasmic reticulum, are relatively stable, whereas those which are closely bound to the microsomal membranes vary greatly in activity according to the structural state of the microsomes[2]. Three enzymatic activities of the latter type, glucose-6-phosphatase (EC 3.1.3.9), an acid inorganic pyrophosphatase, and inorganic pyrophosphate–glucose phosphotransferase have been shown by a variety of methods to be related to each other, possibly properties of the same protein[3–6]. We have studied some of the variations in these three membrane-bound enzymatic activities in response to certain reagents which alter microsomal structure.

METHODS

Except where otherwise described in captions to the figures, the following experimental methods were used.

Final concentrations used for optimal measurements of enzyme activities were 0.08 M glucose 6-phosphate, 0.08 M PP_i, or 0.08 M PP_i with 0.4 M glucose and 0.1 M acetate or 0.08 M sodium cacodylate–HCl buffer. Incubation was at 30° for 10 min and measurements of P_i, glucose 6-phosphate and protein were carried out as previously described[7]. Suitable corrections were made in the calculations of glucose-6-phosphatase and pyrophosphatase activities for the P_i present after parallel incubations in each experiment with microsomal enzyme preparations in which glucose-6-phosphatase had been destroyed by prior incubation for 30 min at 37° (ref. 8). This supplied corrections for pre-existing P_i in substrate and enzyme preparations as well as for very slight contaminating activity of lysosomal acid phosphatase. No correction was necessary for the transferase activity as no glucose 6-phosphate was formed by inactivated enzyme under the assay conditions used.

The pH values recorded were those measured at room temperature on aliquots taken from each reaction mixture at the end of the enzymatic reaction time. In no case did the pH change more than 0.1 of a unit from that of the substrate mixtures alone.

Adult male rats of the Wistar strain, weighing about 250 g, were used. Fasting was for 24 or 48 h. Liver was homogenized with 9 volumes of 0.25 M sucrose containing 1 mM EDTA (pH 7.0). The microsomes used consisted of the fraction which precipitated from the homogenates in 1 h at 105 000 × g after the fraction precipitating in 10 min at 8500 × g had been discarded. Whole homogenates were assayed immediately without freezing. Microsomes were resuspended in 4 ml of isotonic sucrose per ml of liver, stored frozen in small batches and thawed just before use. The protein concentration in assay mixtures was between 1 and 2 mg per ml.

RESULTS

Comparison of stability of enzymatic activities of variously activated microsomes

When freshly thawed microsomes, preincubated in the absence of substrate at 30° in neutral isotonic sucrose containing 1 mM EDTA, were assayed after various time intervals, the three enzymatic activities, glucose-6-phosphatase, inorganic pyrophosphatase, and inorganic pyrophosphate–glucose phosphotransferase, remained parallel and appeared to decrease very slightly over a period of 1 h (Fig. 1A). The identical pretreated microsomal preparation assayed in the presence of approximately optimal concentrations of Triton X-100 or of deoxycholate showed

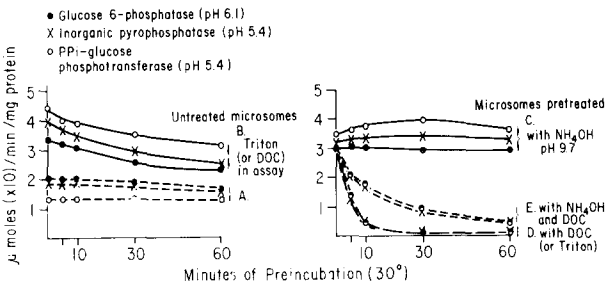

Fig. 1. Stability of the enzyme at 30° in the absence of substrate, as a function of time. Rat-liver microsomes, treated in various ways, were preincubated at 30°. Aliquots were removed at time intervals between 0 and 60 min and assays for glucose-6-phosphatase, inorganic pyrophosphatase and PP_i–glucose phosphotransferase activities were carried out for 10 min at 30° using optimum substrate concentrations, pH 5.4 for inorganic pyrophosphatase and PP_i–glucose phosphotransferase, and pH 6.1 for glucose-6-phosphatase. Microsomes containing about 2 mg of protein were used per ml of assay mixture. All microsomes were from a single 360-g, 9-month-old rat fasted for 24 h. A. Microsomes, stored frozen at pH 6.6 in a suspension equivalent to 0.25 g liver per ml of 0.25 M sucrose containing 0.001 M EDTA, were preincubated immediately after being thawed at 0°. B. Microsomes were stored and treated exactly as in Expt. A, but 0.04% Triton X-100 was included in the enzymatic assay mixtures. Similar results were obtained with 0.02% deoxycholate (DOC). C. Microsomes were stored frozen in sucrose–EDTA as in Expt. A, but with 1 M NH_4OH (pH 10.3) added to a final concn. of 0.1 M NH_4OH and pH 9.7 before freezing. D. Microsomes were stored frozen in sucrose–EDTA as in Expt. A but with 2% (w/v) neutral aqueous deoxycholate added to a final concn. of 0.2% and pH 7.1 before freezing. The deoxycholate was further diluted to 0.02% by addition of the treated enzyme to the assay mixture. E. Microsomes pretreated with NH_4OH before freezing (as in C), were thawed and neutral 2% deoxycholate was added to a final concn. of 0.2% and pH 9.5 before preincubation. Units on the ordinate are glucose-6-phosphate formed for the transferase, and PP_i or glucose 6-phosphate hydrolyzed for the hydrolase activities.

greatly increased enzymatic activity, the initial increase varying from about 50% for glucose-6-phosphatase to more than 300% for the transferase (Fig. 1B). With increasing preincubation time the stimulation effect of the activator in the assay was progressively less and the total amount of each activity was decreased, the relative enzymatic activities remaining parallel. This indicates that the apparent constancy of the activity on heating at 30° was probably due to a fortuitous combination of activation and thermal inactivation.

Treatment of the same microsomal preparation in 0.1 M NH_4OH at pH 9.7 produced a similar increase in the enzymatic activities but the activities remained relatively stable to heating at 30° in the absence of substrate[9] (Fig. 1C). Some in-

crease in activity, especially of the transferase, on incubation at pH 9.7 was often noted. This stability is in marked contrast to the great thermal instability introduced along with activation by treatment of microsomes with Triton X-100 or deoxycholate (Fig. 1D), first observed for glucose-6-phosphatase by BEAUFAY AND DEDUVE[10]. The instability induced is so immediately apparent at 30° that preparations activated with deoxycholate cannot safely be brought to thermal equilibrium prior to addition to substrate solutions, a factor greatly complicating kinetic studies. The thermal inactivation of the microsomal enzymes by surface-active agents proceeds with only slightly less rapidity in the presence of NH_4OH at pH 9.7 (Fig. 1E), the three activities remaining parallel.

The presence of substrate molecules protect the enzymes from heat inactivation[7,8]. However, even in the presence of optimum substrate concentrations, the stability of the microsomal enzymes is greater after exposure to dilute alkaline conditions than after detergent treatment (ref. 9, Fig. 1). In order to obtain optimum activation with a minimum loss of enzyme due to inactivation it is suggested that the levels of activity of these enzymes be measured on NH_4OH-activated microsomes or homogenates rather than on untreated or detergent-treated preparations as has been done in the past[4,5,7,11,12].

Shift in pH optima of enzyme activities in activated microsomes

A number of studies were made of the effect of pH upon the velocity of the three enzymatic activities both before and after various activation procedures. In all cases very high substrate concentrations were employed in an attempt to saturate the available active groups of the enzyme thus eliminating the effect of changes in affinity, that is to study the effect of pH on $v_{max.}$ uncomplicated by possible changes in K_m (ref. 13).

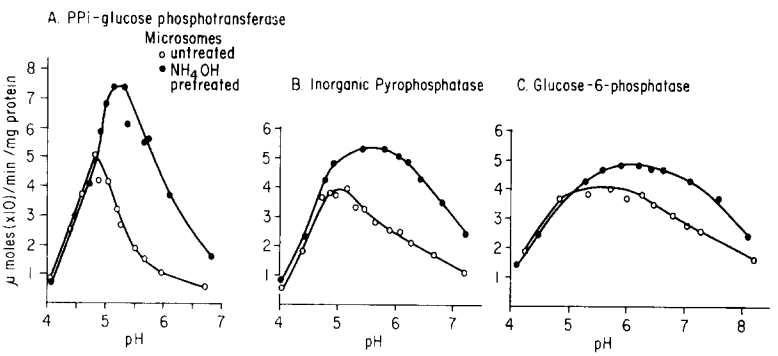

Fig. 2. Effect of pH on the three enzymatic activities of untreated and NH_4OH-pretreated microsomes. The preparation of microsomes used was from the liver of a single 270-g rat fasted for 24 h. Before freezing, one volume of H_2O or of 1 M NH_4OH was added to 9 volumes of a suspension of microsomes in isotonic sucrose containing 1 mM EDTA. Reaction was started by the addition of freshly thawed microsomes to substrate–buffer solutions so that assay concentrations were 0.08 M PP_i and 0.4 M glucose in A, 0.08 M PP_i in B, 0.08 M glucose 6-phosphate in C, each in 0.08 M sodium cacodylate–HCl buffer of the indicated pH's at 30°. Aliquots were removed after 10 min of incubation at 30° and final pH levels were measured immediately on the remainder of the assay mixtures. Units on the ordinate are glucose 6-phosphate formed in A and PP_i and glucose 6-phosphate hydrolyzed in B and C.

A typical set of pH–activity curves for the three enzymatic activities is given in Fig. 2 in which the activities of a single preparation of liver microsomes from a 24-h fasted rat, untreated and pretreated with 0.1 M NH_4OH at pH 9.5, are compared as a function of pH. A marked shift in the apparent pH optima was noted in all cases. The shift was from an optimum of about pH 4.8 to 5.2–5.4 for the transferase,

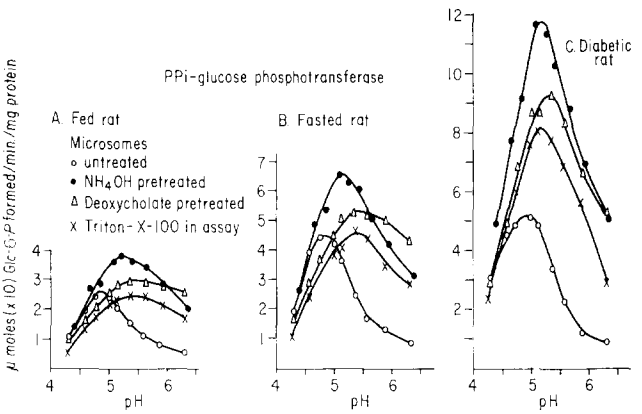

Fig. 3. pH–activity curves for PP_i–glucose phosphotransferase of intact and of variously activated microsomes. Comparison of fed, fasted and diabetic rats. Assay conditions were as described for Fig. 2. For the deoxycholate studies, one volume of 2% deoxycholate (pH 7.6) was added to 9 volumes of suspended microsomes prior to freezing. Where Triton X-100 was used, untreated microsomes were added to reaction mixtures containing 0.04% Triton X-100. The source of microsomes was the liver of a 270-g fed rat for A, of a 230-g litter mate which had been fasted for 48 h for B, and of a 300-g fed diabetic rat for C. Diabetes was induced by the intraperitoneal injection into a fasted rat of 150 mg of alloxan monohydrate per kg. The diabetic rat was killed 10 days after the injection of alloxan at which time it was excreting about 6 g of glucose in a volume of about 100 ml of urine each day.

from 5.0 to about 5.4–5.8 for inorganic pyrophosphatase, and from 5.2–5.6 to 5.8–6.5 for the glucose-6-phosphatase activity. In another study microsomal preparations pre-activated with NH_4OH or deoxycholate or activated instantaneously by the inclusion of Triton X-100 in the assay mixture were compared with the inactivated microsomes (Figs. 3 and 4). Approximately the same shift toward a more alkaline

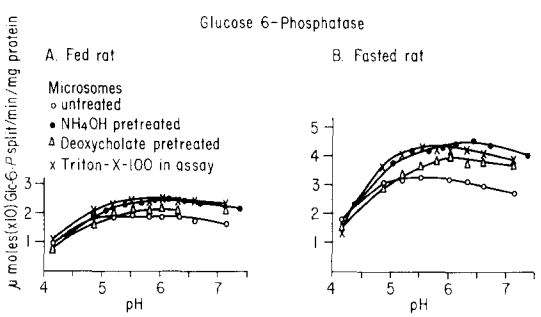

Fig. 4. pH–activity curves for glucose-6-phosphatase of intact and of variously activated microsomes. Comparison of fed and fasted rats. Assay conditions and pretreatment of microsomes were as described for Figs. 2 and 3. The same microsomal preparations from fed and fasted rats were used as in Fig. 3, A and B.

pH optimum was seen to result from activation of the microsomal preparations with the anionic detergent deoxycholate and the non-ionic Triton X-100 as was produced by pretreatment with hydroxyl ion. The relative quantitative activation effects were greatest for the transferase activity and least for glucose-6-phosphatase, the effect on the inorganic pyrophosphatase activity falling in the intermediate range. The consistently higher values obtained with NH_4OH-activated microsomes as compared with those activated by deoxycholate or Triton X-100 is further evidence for greater enzyme stability after NH_4OH treatment. The quantitative results with deoxycholate and Triton X-100 are no doubt a combination of the activation effects and of the greater ease of inactivation induced by the detergent treatment. The similarity of all of the pH–activity curves at very low pH values no doubt is the result of the well-known irreversible inactivation of microsomal glucose-6-phosphatase below pH 5 (ref. 8).

Comparison of effects upon fed, fasted and diabetic rats

Typical pH–activity curves for fed, fasted and diabetic rats are given in Fig. 3 for inorganic pyrophosphate–glucose phosphotransferase and in Fig. 4 for glucose-6-phosphatase. Quantitative levels of activity measured under optimum conditions were nearly twice as high for fasted and about 3 times as high for the diabetic rats as for normal fed rats[5]. In spite of these great quantitative differences the same pH optima and the same shifts in optimum pH values after treatment with NH_4OH, deoxycholate or Triton X-100 were found for the three types of animals studied.

Some analytical consequences of the shift in pH optimum

It is immediately apparent from the results pictured in Figs. 2, 3 and 4 that it is not valid to make comparisons between enzymatic levels in variously treated animals if the assays are carried out at a single pH value for both untreated and activated microsomes. The much narrower pH optimum range for the PP_i–glucose phosphotransferase as well as the relatively greater quantitative effect of detergents on this activity make caution in the interpretation of results more necessary here than in the case of glucose-6-phosphatase. For example, ARION AND NORDLIE[14] compared enzymatic activities in untreated and deoxycholate-treated liver homogenates from fed and fasted rats, determining glucose-6-phosphatase at pH 6.5 and PP_i–glucose phosphotransferase at pH 5.5. They found little difference between transferase levels of unactivated microsomes from fed and fasted rats. Their results led them to conclude that "the enzyme newly formed in response to fasting is principally a phosphohydrolase possessing latent phosphotransferase activity" and to draw physiological conclusions from these analyses.

Fig. 5 represents the results of a typical experiment in which we compared, as a function of pH, the apparent phosphotransferase levels of freshly prepared unfractionated homogenates, untreated or treated with deoxycholate, using conditions of assay as nearly as possible like those of ARION AND NORDLIE[14]. Like those authors, we would have concluded that there was little difference between transferase levels of fed and fasted rat livers had we measured the activities only at pH 5.5 (broken lines, Fig. 5). On the other hand, measured at optimal pH values, it is evident that the enzymatic level of the fasted rat was about twice as high as that of the fed rat

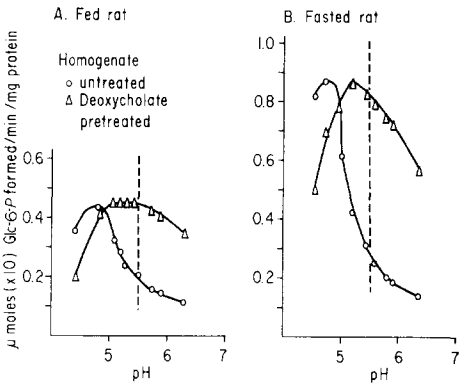

Fig. 5. Inorganic pyrophosphate–glucose phosphotransferase levels in whole homogenates of rat liver with and without added deoxycholate, measured as a function of pH. Liver from a normal 270-g fed red was used in Expt. A, from a 48-h fasted rat in Expt. B. For comparison with assays reported by ARION AND NORDLIE[14] 0.02 M PP_i, 0.18 M glucose and 0.04 M cacodylate buffer were used. Nine volumes of freshly prepared unfractionated homogenate were pretreated at 0° with one volume of either H_2O or of 2% deoxycholate.

regardless of whether or not deoxycholate was employed. In parallel experiments the same homogenate preparations were assayed using the high, optimal substrate concentrations (0.08 M PP_i and 0.4 M glucose) which we regularly use instead of the lower concentrations (0.02 M PP_i and 0.18 M) of ARION AND NORDLIE[14]. Curves very similar to those of Fig. 5 were obtained but the absolute levels of activity were about twice as high.

When the effect of deoxycholate upon the enzyme level is considered in this experiment (Fig. 5), one might conclude for both fed and fasted rats that the transferase activity is increased 2–3-fold by deoxycholate if measured at pH 5.4, unchanged at pH 5.0, or greatly decreased by deoxycholate if measured at pH 4.5. A combination of activation of the microsomal enzyme in the absence of deoxycholate below pH 5, but not at higher pH levels, with increased sensitivity to inactivation by acid conditions after deoxycholate treatment is a likely explanation of the apparent pH optimum shift.

A difference between PP_i–glucose phosphotransferase and glucose-6-phosphatase

Since the original observation by RAFTER[15] that liver particulate fractions were capable of catalyzing the synthesis of glucose 6-phosphate from PP_i and glucose, a number of studies have been interpreted as indicating the probable identity of the enzyme catalyzing this process with the much studied microsomal enzyme glucose-6-phosphatase[3–6]. We have now noted a property in which the transferase and the related PP_i-hydrolyzing activity differ from glucose-6-phosphatase. ASHMORE AND NESBETT[16], in studying the effect of bile acids on glucose-6-phosphatase, observed that slight variations in the concentration of bile acids between $5 \cdot 10^{-4}$ and $1 \cdot 10^{-3}$ M resulted in great quantitative variation in the activity *in vitro* of this predominantly lipid-bound enzyme. Surprisingly they noted that the very low concentrations of about $5 \cdot 10^{-4}$ M caused inhibition while increasing concentrations up to $1 \cdot 10^{-3}$ M resulted in accelerated activity, still higher concentrations causing

inactivation along with solubilization. We have confirmed this pattern of behavior for glucose-6-phosphatase with deoxycholate and found that the same initial inhibition followed by acceleration of activity obtains with increasing concentrations of such unphysiological surface-active agents as Triton X-100 as well (Fig. 6). On the other hand similar concentrations of deoxycholate or Triton X-100, acting on the identical microsomal preparations resulted only in stimulation of the activity of PP_i–glucose phosphotransferase and inorganic pyrophosphatase as the detergent concentration was increased to an optimal level. No inhibition of these activities was observed at low detergent concentrations. As the concentrations were increased, inactivation of the transferase always occurred at lower detergent concentrations than those causing inactivation of the hydrolases. The curves obtained in experiments using increasing concentrations of deoxycholate instead of Triton X-100 were nearly identical except that the instability of the enzyme was more evident at

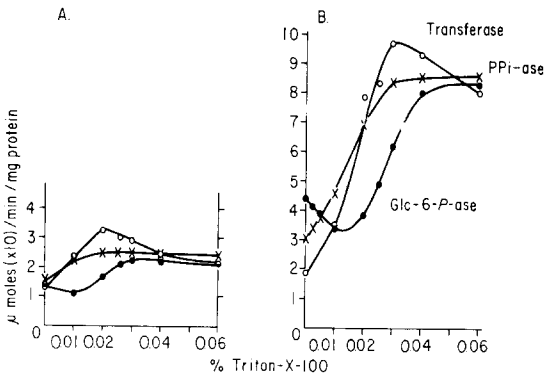

Fig. 6. Comparison of enzymatic activities assayed at various levels of Triton X-100. Normal and diabetic rats. Enzymatic reactions were started by the addition of untreated microsomes to substrate–buffer mixtures containing different levels of Triton X-100. Substrate concentrations used were 0.08 M glucose 6-phosphate, 0.08 M PP_i, or 0.08 M PP_i and 0.4 M glucose in 0.1 M acetate buffer at pH 5.4. In Expt. A liver microsomes were from a normal fed 250-g rat; those in Expt. B were from a comparable alloxan diabetic rat.

high concentrations of deoxycholate than of Triton X-100. The observed difference between glucose-6-phosphatase on the one hand and inorganic pyrophosphatase and transferase on the other persisted when citrate or cacodylate buffers were used in place of acetate and when glucose-6-phosphatase was assayed at pH 6.5 instead of 5.4.

The phenomenon of inhibition of glucose-6-phosphatase without parallel inhibition of inorganic pyrophosphatase and transferase at low concentrations of deoxycholate or Triton X-100 was repeatedly confirmed with variously treated rats. Typical results for a normal fed rat with low enzyme levels are plotted in Fig. 6A and for an alloxan diabetic rat with a very high concentration of enzyme activity in Fig. 6B. Curves with intermediate enzyme levels between these extremes but with the same maxima and minima and the same kind of difference between glucose-6-phosphatase and the other activities were also obtained with microsomes from fasted and from cortisone-treated rats.

DISCUSSION

A number of possible hypotheses may be evoked to explain the elevation *in vitro* in activities of insoluble enzymes which occur bound to or as an integral part of microsomes. Conformational changes in the membranes such as unfolding or breaking of interfering bonds can be pictured as making active sites on the protein more readily accessible to substrate molecules. Alternatively the possibility exists that the limited access of the substrate to active sites is a result of the artificial fragmentation of the endoplasmic reticulum into microsomal particles during homogenization of the tissue and that the action of detergent or of OH^- tends to restore activity to a state more nearly approaching that found in the intact cells. However, if the picture proposed by ERNSTER, SIEKEVITZ AND PALADE[2] is accepted, microsomes may be pictured as small vesicles representative of the intact endoplasmic reticulum, with outer and inner surfaces of the membrane surrounding a portion of the original solution of the lumina. The action of such agents as deoxycholate results in separation of ribosomes from the microsomal membranes[1] and by analogy with the effects on mitochondria[17], probably causes rupture of lipid–lipid or of lipid–protein bonds resulting in some fragmentation and opening up of microsomal particles. Perhaps ribosomal particles are attached to the membranes in such a way as to prevent access of substrate molecules to suitable sites of binding.

We have consistently found that the potential for synthesis of glucose 6-phosphate from PP_i and glucose is more restricted than is that for glucose 6-phosphate hydrolysis in whole untreated microsomal preparations. A much greater degree of activation is observed for the PP_i–glucose phosphotransferase than for the glucose-6-phosphatase activity of liver microsomes by reagents which alter microsomal membranes. This observation is consistent with the hypothesis that this multi-functional enzyme is so oriented within the endoplasmic reticulum membrane that the active binding sites for glucose 6-phosphate are predominently on the outer surface of the microsomes, that is, readily accessible to the cytoplasm of the cell, while the sites for glucose are largely on that inner surface which corresponds to the space between the ergastoplasmic membranes and are, in effect, outside of the cell. DEDUVE has suggested the possibility that glucose 6-phosphatase is so oriented in the membrane as to permit direct secretion or excretion of the products of glucose 6-phosphate hydrolysis*. Our observations support this idea and also lead to the suggestion that the sites for binding of glucose are chiefly located on the membrane surface within the endoplasmic reticulum lumen with the enzyme oriented in such a way as to permit synthesis from PP_i and glucose to channel the glucose 6-phosphate formed into the true intracellular compartment. It may well be significant that the enzyme activities under consideration are found exclusively in those organs, liver, kidney, and small intestine which are involved in active transport of sugars between intracellular and extracellular compartments.

A satisfactory explanation for the effects of various reagents in activating and inactivating enzymes which occur bound to or as integral parts of the endoplasmic reticulum must certainly await a better basic understanding of the nature of the phospholipid–protein bonds in these membranes.

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