Biochemical and cytochemical localization of inosine-5'-diphosphatase in rat liver microsomal fractions

James S. LITTLE

Department of Clinical Investigation, Madigan Army Medical Center, Tacoma, WA 98431, U.S.A.

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A procedure has been developed for the biochemical and cytochemical localization of inosine-5'-diphosphatase (IDPase) in rat liver microsomal fractions. The concentration of Pb^{2+} ions used in the cytochemical incubation medium inhibited IDPase by 32%. IDP formed during the incubation was not hydrolysed further to inosine, and no significant non-enzymic hydrolysis of substrate occurred. When microsomal fractions were incubated cytochemically for IDPase and separated from unchanged substrate under conditions such that the microsomal membrane was not permeable to EDTA, approx. 80% of the reaction product was solubilized by EDTA. In the electron microscope, lead precipitate was observed on the cytoplasmic side (outside) of a major fraction of the vesicles and on the cisternal side (inside) of others. After extraction with EDTA, the lead precipitate was observed only on the cisternal side. Since it was shown that phosphate is trapped on the same side of the membrane as it is released, it is concluded that IDPase can release phosphate on either the cisternal or the cytoplasmic side of the microsomal membrane.

Cytochemical techniques based on the Gomori lead phosphate procedure have been used to localize phosphatases in intact tissue (Goldfischer *et al.*, 1964, 1971; Farquhar *et al.*, 1974) and in isolated cell fractions (Marchesi & Palade, 1967; Leskes *et al.*, 1971b; Farquhar *et al.*, 1974). Membrane-bound phosphatases are generally localized asymmetrically, so that reaction product is observed only on one side of the membrane.

When microsomes (microsomal fractions) are incubated cytochemically for G6Pase, reaction product (lead phosphate) is localized on the cisternal side of the membrane and is not solubilized by EDTA (Leskes *et al.*, 1971b; Widnell, 1972). In contrast, reaction product from 5'-nucleotidase (EC 3.1.3.5) is localized on the cytoplasmic side of the microsomal membrane and is completely solubilized by EDTA (Widnell, 1972).

It therefore seemed likely that, for any microsomal phosphatase, the fraction of lead phosphate solubilized by EDTA should equal the fraction of reaction product on the cytoplasmic side of the membrane. Since a quantitative estimation of this type is virtually impossible to obtain from electron micrographs, it was decided to test this hypothesis

by studying the localization of IDPase in isolated microsomes.

Novikoff & Heus (1963) have shown that a nucleoside diphosphate (IDPase) that hydrolyses IDP, UDP and GDP is present in the endoplasmic reticulum. Reaction product from IDPase is observed on the cisternal side of the endoplasmic reticulum *in situ* (Goldfischer *et al.*, 1971), and biochemical experiments with isolated microsomes have been interpreted to support this localization (Kuriyama, 1972). The purified enzyme does not hydrolyse ADP (Kuriyama, 1972).

In preliminary experiments with isolated microsomes, it was observed, contrary to expectation, that most of the reaction product from IDPase was solubilized by EDTA. Therefore biochemical controls were used to develop a cytochemical procedure for the localization of this enzyme in isolated rat liver microsomes. It is reported here that about 80% of the reaction product from IDPase is solubilized by EDTA and that a major fraction of this lead precipitate is localized on the cytoplasmic side of the membrane.

Materials and methods

Materials

Nucleotides were obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.) and [3H]IDP

Abbreviation used: G6Pase, glucose-6-phosphatase (EC 3.1.3.9).

(4.0 Ci/mmol) was from Amersham/Searle Corp. (Arlington Heights, IL, U.S.A.). The barium salt of glucose 6-phosphate was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), converted into the free acid by using a Dowex-50 (H^+ form; Dow Chemical Co., Midland, MI, U.S.A.) ion-exchange column, and neutralized with $NaHCO_3$ before use.

Animals

Male Sprague-Dawley rats weighing 250–300 g were used for all experiments. All animals were starved for 14–16 h before experimentation.

Cell fractionation

Total microsomes were isolated from rat livers and characterized biochemically as previously described (Little, 1977). Isolated microsomes were washed by suspension (1.5 mg of protein/ml) and re-centrifuged at 105 000 g for 45 min in 0.25 M-sucrose/20 mM-Tris/acetate, pH 7.5, to remove absorbed cytoplasmic proteins (Dallner *et al.*, 1966).

Biochemical enzyme assays

G6Pase was assayed by a modification of the methods of Swanson (1950) and de Duve *et al.* (1949) as previously described (Little, 1977). 5'-Nucleotidase was assayed by the method of Widnell (1972). IDPase was assayed as described (Little *et al.*, 1976) by a modification of the method of Ernster & Jones (1962).

Analytical procedures

Free and total P_i were assayed by the procedure of Chen *et al.* (1956). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Concentrations of all nucleotides were determined as described by Bock *et al.* (1956).

Cytochemical enzyme assays

For IDPase, incubations were performed in 20 ml containing 0.5 mM-IDP, 0.5 mM- $Pb(NO_3)_2$, 50 mM-Tris/acetate, pH 7.2, and microsomes at a concentration such that no more than 30% of the substrate was hydrolysed during a 20 min incubation at room temperature. Reactions were stopped by cooling to approx. 2°C in a salt/ice bath, and the microsomes sedimented by centrifugation at 30 000 g for 15 min. The pellet was resuspended with a ground-glass homogenizer in 5 ml of 0.25 M-sucrose/10 mM-Tris/acetate, pH 6.6, layered over either 20 ml of 30% (w/v) Ficoll/10 mM-Tris/acetate, pH 6.6, or a discontinuous sucrose gradient (Widnell, 1972), and centrifuged in a swinging-bucket rotor at 40 000 g for 90 min. The pellet was rinsed twice with water, resuspended in 15 ml of

water, centrifuged at 10 000 g for 15 min and resuspended as described below for biochemical or electron-microscopic analysis.

Incubations for G6Pase were performed in 8 ml containing 1 mM-glucose 6-phosphate, 1.5 mM- $Pb(NO_3)_2$, 100 mM-Tris/maleate, pH 6.6, and microsomes at a concentration such that not more than 30% of the glucose 6-phosphate was hydrolysed during a 20 min incubation at room temperature. Reactions were stopped and the microsomes obtained as a pellet as described above.

Electron microscopy

Microsomes were prepared as described above and suspended in 0.1 M-sodium cacodylate (pH 7.2)/10 mM- $Mg(NO_3)_2$. Then 1 ml of 2% (w/v) OsO_4 was added to 1.0 ml of this suspension, and after 3 h at 4°C the microsomes were obtained as a pellet by centrifugation for 30 min at 50 000 rev./min in a Spinco SW 50 rotor. The intact pellet was dehydrated in a series of graded ethanol, followed by propylene oxide and embedded in Epon/Araldite. Sections through the depth of the pellet were cut on a Porter-Blum microtome, stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined with a Philips EM 300 electron microscope.

Solubilization of reaction product by EDTA

Microsomes that had been incubated in the cytochemical medium for G6Pase or IDPase were pelleted by centrifugation at 30 000 g for 15 min. The pellets were suspended with a ground-glass homogenizer in 0.25 M-sucrose/10 mM-Tris/acetate, pH 7.5. A portion of this suspension was deproteinized with 10% trichloroacetic acid and assayed for P_i .

Samples containing 1.0 μ mol of P_i were suspended to a final volume of 3.0 ml in 0.25 M-sucrose/10 mM-Tris/acetate (pH 7.5)/1 mM-EDTA. After 30 min at 2°C the suspensions were centrifuged for 10 min at 30 000 g and P_i was analysed in the supernatant and pellet fractions after deproteinization with 10% trichloroacetic acid.

Characterization of reaction products from IDPase

The cytochemical incubation for IDPase was performed in the presence of 12.5 μ Ci of [3H]IDP. At appropriate time intervals, samples were deproteinized in the cold with $HClO_4$, neutralized with $KHCO_3$ and centrifuged to remove the salt. A sample of the supernatant was subjected to high-voltage electrophoresis (Camag) at 100 V/cm for 45 min on Whatman no. 1 paper in pyridine/acetate buffer, pH 3.5 (pyridine/acetic acid/water, 1:10:292, by vol.). After drying, the reaction products were detected with u.v. light; the electrophoretogram was cut into 1.27 cm ($\frac{1}{2}$ in) strips, each

Fig. 1. Effect of Pb^{2+} concentration on the activity of microsomal G6Pase (■) and IDPase (●)

All reactions were carried out at 22°C. The incubation medium for G6Pase contained 100mM-Tris/maleate, pH6.6, and 1mM-glucose 6-phosphate. For IDPase the incubation medium contained 0.5mM-IDP, 50mM-Tris/acetate, pH7.2, and 1.0mM-Mg(NO₃)₂. The specific activities (μ mol of P_i released/30 min per mg of protein) of the controls incubated without Pb^{2+} were: G6Pase, 0.885; IDPase, 0.815.

was placed in 10ml of Bray's solution, and the radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer.

Results

Cytochemical incubation media

The routine assay for IDPase was performed at 37°C in a medium containing 5mM-IDP, 4mM-MgCl₂ and 50mM-Tris/HCl, pH7.5. In preliminary experiments designed to determine conditions necessary to avoid formation of non-specific lead deposits, it was found that best results were obtained by lowering the IDP concentration to 0.5mM and by substituting 1mM-Mg(NO₃)₂ for 4mM-MgCl₂ and Tris/acetate, pH7.2, for Tris/HCl, pH7.5. Also, the structure of the unfixed microsomes was improved by incubation at room temperature rather than at 37°C.

The routine assay for G6Pase contained 10mM-glucose 6-phosphate and 100mM-Tris/maleate, pH6.6. Best results for cytochemical studies were obtained by using 1mM-glucose 6-phosphate,

Table 1. Characterization of reaction products from IDPase and determination of the non-enzymic hydrolysis of IDP

Cytochemical incubations were performed in 20ml as described in the Materials and methods section. Incubation mixtures for IDPase contained [³H]IDP (12.5 μ Ci). For P_i determination, microsomes were made to react in the cytochemical medium, separated from unchanged substrate by the Ficoll procedure, deproteinized and assayed for P_i as described in the Materials and methods section. Incubation mixtures that did not contain microsomes were centrifuged at 10000g for 10min at the end of the reaction, and P_i was determined in the pellet. For determination of inosine and IMP formed during the reaction, a sample of the reaction mixture that had been chilled to 2°C immediately after the reaction was made 7% in HClO₄, neutralized with KHCO₃ and centrifuged (3000g for 10min) to remove the salt. A sample of the supernatant was spotted on Whatman no. 1 paper, subjected to high-voltage electrophoresis and analysed as described in the Materials and methods section. Since the recovery of radioactivity from the strips was essentially quantitative, the radioactivity in inosine, IMP and IDP was calculated as percentages of the total radioactivity. Each of the values represents the mean of at least two experiments.

Microsomes	P_i trapped (μ mol)	Inosine released (μ mol)	IMP released (μ mol)
—	0.15	0	0.20
+	2.10	0.204	2.30

1.5mM-Pb(NO₃)₂ and 100mM-Tris/maleate, pH6.6.

When microsomes were assayed for glucose-6-phosphatase in this cytochemical system, lead phosphate precipitates were observed only on the cisternal side of microsomal vesicles and were essentially insoluble in EDTA. These results are in agreement with those previously published (Leskes *et al.*, 1971b; Widnell, 1972).

Pb^{2+} ions have been shown to inhibit several phosphatases (Novikoff *et al.*, 1958; Marchesi *et al.*, 1972). The effect of Pb^{2+} concentration on microsomal IDPase and G6Pase is shown in Fig. 1. Pb^{2+} inhibited IDPase at all concentrations: at 0.5mM-Pb(NO₃)₂, IDPase was inhibited by approx. 32%. Pb^{2+} at concentrations studied here had no effect on G6Pase activity. This observation agrees with that by Leskes *et al.* (1971a).

Characterization of the reaction products from IDPase

To localize IDPase, it was necessary to determine whether the products of the reaction were only P_i and IMP or whether the IMP formed was

Table 2. *Effect of Pb^{2+} concentration and the presence of organic diphosphates or inosine on the solubilization of the reaction product from G6Pase by EDTA*

All reactions were performed for 20 min at 22°C in 10 ml containing 50 mM-Tris/acetate, pH 7.2, 1.0 mM- $Mg(NO_3)_2$, 0.5 mM substrate, and the indicated Pb^{2+} concentration. After the reaction, the incubation mixtures were chilled to about 2°C and centrifuged at 10000g for 15 min. The pellets from this centrifugation were suspended (approx. 1 mg of protein/ml) in 0.25 M-sucrose/10 mM-Tris/acetate, pH 6.6, with a ground-glass homogenizer and layered over 30% (w/v) Ficoll containing 10 mM-Tris/acetate, pH 6.6. Recovery from the gradients and solubilization of P_i by EDTA were determined as described in the Materials and methods section. In all cases at least 95% of the P_i released was trapped as lead phosphate. Each value represents the mean of at least two experiments. Values in parentheses represent the fraction of reaction product from G6Pase solubilized by EDTA after correction was made for the release of P_i from either TPP or dTDP. Abbreviations: G6P, glucose 6-phosphate; TPP, thiamin pyrophosphate; dTDP, thymidine diphosphate.

Substrate(s)	Pb^{2+} concn. (mM)	P_i trapped (μ mol)	P_i solubilized by EDTA	
			(μ mol)	(%)
G6P	0.5	1.25	0.138	11.0
G6P	1.5	1.50	0.183	12.2
TPP	0.5	0.143	0.143	100
G6P + TPP	0.5	1.46	0.256	17.5 (8.5)
dTDP	0.5	0.117	0.115	98.9
G6P + dTDP	0.5	1.32	0.308	23.4 (16.0)
G6P + inosine	0.5	1.11	0.174	15.7
IDP, then G6P*	0.5	1.11	0.180	16.2

* This sample was first made to react for IDPase in the standard cytochemical medium, but without $Pb(NO_3)_2$. After incubation, the membranes were separated from the medium by centrifugation, suspended in water, and then made to react for G6Pase as described above.

hydrolysed in turn by 5'-nucleotidase, which is also present in microsomal membranes (Widnell, 1972). Incubations were therefore performed with [3H]IDP, and the products of the reaction were characterized by high-voltage electrophoresis. As shown in Table 1, IMP and P_i were formed in chemically equivalent amounts, and less than 10% of the IMP formed was hydrolysed to inosine. This result is in agreement with the report (Sullivan & Alpers, 1971) that nucleoside diphosphates are potent inhibitors of 5'-nucleotidase. Table 1 also shows that there was very little non-enzymic hydrolysis of IDP during the incubation.

Separation of microsomes from unchanged substrate

After incubation in the cytochemical medium, it was necessary to separate microsomes from unchanged substrate, since in the electron microscope complexes of lead and substrate might be indistinguishable from lead phosphate precipitates. To determine the localization of reaction product by estimating the solubility of lead phosphate in EDTA, it was also essential that any procedure used to remove unchanged substrate from microsomes had no effect on the permeability of the microsomal membrane to EDTA.

[3H]IDP was employed to determine the binding of unchanged substrate to microsomes. The solubilization of reaction product from G6Pase by EDTA was used to estimate the permeability of microsomal membranes to EDTA. Although centrifugation through discontinuous sucrose gradients dissociated over 91% of the unchanged IDP

from microsomes, this treatment also increased the permeability of microsomal membranes to EDTA approx. 5-fold. It is likely that the association of lead phosphate with the microsomes alters their osmotic properties, since this change was not observed when microsomes were centrifuged through sucrose before cytochemical incubation for G6Pase.

When microsomes were sedimented through 30% (w/v) Ficoll after cytochemical incubation for IDPase, only 1.4% of the unchanged IDP remained associated with the microsomes. This procedure caused essentially no change in the permeability of the microsomal membrane to EDTA and was therefore employed for subsequent biochemical analysis.

Relationship between the site of P_i release and the site of lead phosphate precipitation

Microsomal membranes are freely permeable to Pb^{2+} ions and P_i (Nilsson *et al.*, 1973). It was therefore possible that P_i might cross the microsomal membrane before precipitation as lead phosphate. This could occur either because of the inherent permeability of the microsomal membrane or as a result of the formation of a complex between Pb^{2+} ions and the nucleoside diphosphate substrate, which might have decreased their effective concentration.

When microsomes are incubated in a cytochemical medium in the presence of β -glycerophosphate and alkaline phosphatase purified from *Escherichia coli*, the P_i released does not cross the microsomal

Fig. 2. *Electron micrograph of microsomes incubated in the cytochemical medium for IDPase*

Microsomes were incubated for 20 min in the cytochemical medium for IDPase. A typical field is shown which shows that reaction product is observed on either the cisternal side (short arrow) or the cytoplasmic side (long arrow) of the vesicles. The bar represents 0.2 μm . Magnification $\times 87500$.

membrane before precipitation as lead phosphate. In these experiments lead phosphate precipitates were observed only on the cytoplasmic side of a small number of microsomes (J. S. Little & C. C. Widnell, unpublished work).

It was also possible that the pyrophosphate moiety of the nucleotide might decrease the efficiency of lead phosphate precipitation. To test this, incubations were carried out in the cytochemical medium for IDPase, except that glucose 6-phosphate replaced IDP as substrate, and either dTDP or thiamin pyrophosphate was also added to the medium. The amount of lead phosphate solubilized by EDTA was then determined to estimate quantitatively the maximum fraction of the reaction product from G6Pase that was localized on the cytoplasmic side of the microsomal membranes. When the Pb^{2+} concentration in the incubation medium was decreased from 1.5 to 0.5 mM, the amount of P_i trapped and the fraction of lead phosphate solubilized by EDTA were essentially unchanged (Table 2). Under these experimental conditions, there was very little release of P_i from either dTDP or thiamin pyrophosphate, and essentially all of this was solubilized by EDTA. When incubations were carried out in the presence of

glucose 6-phosphate and either dTDP or thiamin pyrophosphate, 23.4% or 17.5% of the reaction product, respectively, was solubilized by EDTA. When a correction was made for the background incubations from which glucose 6-phosphate was omitted, it was clear that these substances had little or no effect on the localization of reaction product from G6Pase.

Table 2 also shows that neither the presence of inosine in the medium nor preincubation of microsomes with IDP caused any change in the amount of reaction product from G6Pase that was solubilized by EDTA.

Localization of reaction product from IDPase

The experiments described above established the procedure for the localization of IDPase. When microsomes were incubated in the cytochemical medium and separated from unchanged substrate by centrifugation through Ficoll, 80% of the reaction product was solubilized by EDTA. These values suggest that only 20% of the reaction product from IDPase is localized on the cisternal side of the microsomal membrane.

When similar preparations were examined in the electron microscope, a heterogeneous distribu-

Fig. 3. *Electron micrograph of microsomes incubated in the cytochemical medium for IDPase*

Microsomes were incubated for 20 min in the cytochemical medium for IDPase. The photograph shows a field selected to demonstrate reaction product on the cisternal side of the membrane (arrow). The bar represents 0.1 μm . Magnification $\times 175000$.

tion of lead phosphate precipitates was observed. Some vesicles were stained densely, whereas others were hardly stained at all. The major fraction of the reaction product seemed to be localized on the cytoplasmic side of the membrane, with the remainder on the cisternal side (Fig. 2). Vesicles were occasionally observed with reaction product on both sides of the same membrane. The preparations were examined at a higher magnification so that the membranes could be observed more clearly. Figs. 3 and 4 show reaction product from IDPase on the cisternal and cytoplasmic sides of the microsomal membrane. Fig. 5 shows reaction product on the cytoplasmic side of what appears to be a ruptured vesicle. In this case there could be no permeability barrier for substrate or trapping agent, and still no reaction product was seen on the cisternal side of this vesicle. After extraction of lead phosphate with EDTA, reaction product was observed only on the cisternal side of the membrane (Fig. 6).

Fig. 4. *Electron micrograph of microsomes incubated in the cytochemical medium for IDPase*

Microsomes were incubated for 20 min in the cytochemical medium for IDPase. Field selected to demonstrate reaction product on the cytoplasmic side of the membrane (arrow). The bar represents 0.1 μm . Magnification $\times 175000$.

Control incubations in the absence of substrate contained little or no lead precipitate associated with the membranes. These microsomes appeared identical with those observed by Widnell (1972) after treatment in the same manner.

Effect of fixation by glutaraldehyde on IDPase activity

Goldfischer *et al.* (1971), using glutaraldehyde-fixed tissue slices, localized IDPase on the cisternal side of the endoplasmic reticulum. Since the localization of IDPase determined here was in apparent conflict with those studies, the effect of glutaraldehyde fixation on IDPase activity was investigated.

Glutaraldehyde treatment caused an inhibition of the enzyme at all concentrations tested. After exposure to glutaraldehyde (0.25–3.0%), only 20% of the enzyme activity remained. Fixed microsomes were made to react in the cytochemical medium for IDPase and examined in the electron microscope. The distribution of reaction product was similar to that in unfixed controls, except that a greater fraction of the reaction product seemed to be localized

Fig. 5. Electron micrograph of microsomes incubated in the cytochemical medium for IDPase

Microsomes were incubated for 20 min in the cytochemical medium for IDPase. Field selected to demonstrate an apparently ruptured vesicle, with reaction product on the cytoplasmic side of the membrane (arrow). The bar represents $0.1\mu\text{m}$. Magnification $\times 175000$.

on the cisternal side of the membrane. Although this suggested that there might be a preferential inactivation of the fraction of the enzyme that released P_i on the cytoplasmic side of the membrane, it was virtually impossible to quantify the results.

An attempt was therefore made to estimate the fraction of the reaction product on the cisternal side of the membrane by its insolubility in EDTA. To determine the effect of fixation on the permeability of the microsomal membrane to EDTA, microsomes were first made to react in a cytochemical medium for G6Pase, fixed with increasing concentrations of glutaraldehyde as described in the legend to Table 3, and finally treated with EDTA. The fraction of the lead phosphate solubilized increased from 10 to 42% (Table 3), which suggested that fixation did increase the permeability of the membrane to EDTA.

When microsomes were first fixed in 1% glutaraldehyde, then made to react in a cytochemical medium for IDPase, and finally treated with EDTA, the fraction of reaction product that was

insoluble increased from 20% in controls to 35%. When a correction was made for the increased permeability of the microsomal membrane to EDTA after fixation, as much as 53% of the reaction product from IDPase was apparently localized on the cisternal side of the membrane. This result also suggests that the fraction of the enzyme that released P_i on the cytoplasmic side of the membrane was preferentially inhibited by fixation.

Discussion

These experiments were designed to test the hypothesis that the fraction of P_i released by any phosphatase on the cytoplasmic side of the microsomal membrane could be determined by estimating the fraction of lead phosphate (reaction product) solubilized by EDTA. It has been demonstrated biochemically that about 80% of the reaction product from IDPase was solubilized by EDTA, and by electron microscopy that a major fraction of the reaction product was localized on the cytoplasmic side of the microsomal membrane.

In addition, the results emphasized that a comprehensive series of biochemical controls were required for such experiments. It has been determined that:

- (1) there was essentially no non-enzymic hydrolysis of substrate during the cytochemical incubation;
- (2) the products of the hydrolysis of IDP were IMP and P_i ;
- (3) the microsomes, which were examined both biochemically and electron microscopically for the localization of reaction product, were free of unchanged substrate;

- (4) the microsomal preparation employed for the estimation of the fraction of reaction product solubilized by EDTA was impermeable to EDTA.

The conclusion that P_i is released from IDPase on the cisternal side of some microsomal vesicles and on the cytoplasmic side of others is only valid if P_i is precipitated as lead phosphate on the same side of the membrane as it is released. This was supported by the following observations.

- (1) The localization of reaction product from IDPase and G6Pase was the same when the Pb^{2+} concentration was varied between 0.5 and 1.5 mM.

- (2) Phosphate released by the action of alkaline phosphatase from *E. coli* on β -glycerophosphate in the presence of microsomes was precipitated as lead phosphate on the cytoplasmic side of the membrane (J. S. Little & C. C. Widnell, unpublished work), even though microsomal membranes are freely permeable to Pb^{2+} and P_i (Nilsson *et al.*, 1973).

- (3) The addition of thiamin pyrophosphate or dTDP did not prevent the formation of reaction

Fig. 6. *Electron micrograph of microsomes incubated in the cytochemical medium for IDPase*
Microsomes were incubated for 20 min in the cytochemical medium for IDPase, isolated by the Ficoll procedure, and then extracted with EDTA. A typical field is shown which shows that reaction product is observed almost exclusively on the cisternal side of the vesicles. The bar represents 0.2 μ m. Magnification $\times 87500$.

product from G6Pase on the cisternal side of the membrane. This suggested that the pyrophosphate moiety of the substrate did not cause a functional decrease in the Pb^{2+} concentration, which would result in diffusion of P_i from the side of release before precipitation.

Goldfischer *et al.* (1971), using cytochemical techniques, suggested that IDPase is localized only on the cisternal side of the endoplasmic reticulum. Although our results are in apparent conflict with their observation, there are two possible reasons why they did not observe reaction product on the cytoplasmic side of the membrane. First, it appears that this fraction of IDPase is preferentially inactivated by glutaraldehyde fixation, and second, lead precipitates on the cytoplasmic side of the membrane are generally more diffuse (Fig. 2, and Goldfischer *et al.*, 1971), so they might be more difficult to distinguish against the background of cytoplasm in the intact cell.

It is also possible that a reorganization of the enzymes in the membrane takes place during homogenization and/or subsequent isolation of the microsomes. This would seem less likely, since reaction product from G6Pase is localized entirely on

the cisternal side of the membrane both in the intact cell (Leskes *et al.*, 1971a) and in isolated microsomes (Leskes *et al.*, 1971b).

It may be possible that the enzyme localized on the cytoplasmic side of microsomal vesicles represents an absorbed enzyme. This seems unlikely, since these microsomal preparations were washed at pH 7.5, a procedure that has been shown to release absorbed cytoplasmic proteins from microsomes (Dallner *et al.*, 1966) and plasma membranes (Little, 1981). In addition, Ernster & Jones (1962) have shown that there is very little, if any, soluble IDPase in rat liver. Essentially all the activity was found in the microsomal fraction.

It might also be proposed that the IDPase that is localized on the cytoplasmic side of microsomal vesicles may be due to contamination by either plasma-membrane vesicles or bile canaliculi. This also seems unlikely, since essentially identical results were obtained in purified rough microsomes, which are not contaminated by these plasma-membrane constituents. In addition, vesicles were occasionally seen which contained reaction product on both sides of the same vesicle.

Kuriyama (1972) has shown that γ -globulin, con-

Table 3. *Effect of glutaraldehyde fixation on the permeability of microsomal membranes to EDTA*

Microsomes were prepared and made to react in the cytochemical incubation medium for G6Pase as described in the Materials and methods section. After the cytochemical incubation, the microsomes were centrifuged for 15 min at 30000g. The microsomes designated as controls were suspended in 4 ml of 0.25M-sucrose/10mM-Tris/acetate, pH 7.5. For fixation, the microsomes that had reacted were fixed by suspension for 1 h at 0–4°C in 5 ml (approx. 1 mg of protein/ml) of 100mM-sodium cacodylate, pH 7.2, which contained glutaraldehyde at the desired concentration. After fixation, the suspensions were centrifuged for 10 min at 10000g. Pellets were washed twice by suspension and resedimentation in 15 ml of 10mM-sodium cacodylate, pH 7.2, and finally suspended in 4 ml of 0.25M-sucrose/10mM-Tris/acetate, pH 7.5. Control and fixed microsomes were then extracted with EDTA as described in the Materials and methods section. Each value represents the mean of at least two experiments.

Glutaraldehyde (%, v/v)	P _i trapped (μmol)	P _i solubilized by EDTA	
		(μmol)	(%)
0 (control)	2.41	0.241	10
0.05	2.40	0.600	25
0.15	2.42	0.678	28
0.25	2.44	0.780	32
0.50	2.43	0.924	38
1.00	2.38	1.09	42

taining antibody against purified IDPase, inhibits about 60% of the enzymic activity in isolated microsomes and approx. 100% of the activity after solubilization in deoxycholate. This could suggest that a major fraction of IDPase is accessible from the cytoplasmic side of the membrane. It was observed (J. S. Little & C. C. Widnell, unpublished work) that γ -globulin containing antibody against purified 5'-nucleotidase inhibited about 90% of the enzymic activity in smooth microsomes either before or after solubilization in deoxycholate. This is consistent with the observation that essentially all the P_i from 5'-nucleotidase is released on the cytoplasmic side of the membrane.

It is conceivable that microsomal membranes might contain a variety of phosphatases that hydrolyse IDP, so that the distribution of reaction product that was observed could reflect the localization of different enzymes. However, during purification, IDPase appears to behave as a single protein, and antibody to purified IDPase inhibits the activity of the enzyme quantitatively in detergent-solubilized microsomes (Kuriyama, 1972). IDPase and ADPase are probably separate enzymes, since purified IDPase does not hydrolyse ADP (Kuriyama, 1972; Novikoff & Heus, 1963). Since the

activity of IDPase is approx. 1.5 times that of ADPase, it is unlikely that reaction product on the cisternal side of the membrane with IDP as substrate could result from the action of ADPase on IDP. It is reasonable to conclude that IDPase releases P_i on the cisternal side of some membranes and the cytoplasmic side of others.

In general, membrane proteins are localized asymmetrically. This conclusion is supported by studies involving the binding of lectins and antibodies (Nicolson *et al.*, 1971), by biochemical analysis (Bretscher, 1971; Marchesi *et al.*, 1972) and by cytochemical localization (Farquhar *et al.*, 1974; Marchesi & Palade, 1967), using essentially the same procedures as employed here. These results for IDPase suggest that asymmetry is not an obligatory feature of the organization of proteins in membranes. Although it has been assumed for many years that proteins involved in membrane transport are accessible from both sides of the membrane, the functional significance of these results remains to be established.

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