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Distribution and Partial Purification of a Liver Membrane Protein Capable of Inactivating Cytosol Enzymes

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1. The inactivation of cytosol enzymes in liver extracts was carried out by several subcellular fractions, with plasma membranes having the highest specific activity. Rough and smooth microsomal fractions were both active, whereas lysosomal inactivation capacity appeared to be derived entirely from contaminating plasma-membrane fragments. 2. Inactivation capacity in liver fractions was derived from parenchymal cells. Of the non-liver cells tested, plasma membranes from H35 hepatoma cells were able to inactivate glucose 6-phosphate dehydrogenase (EC 1.1.1.49), adipocyte 'ghosts' showed slight activity and erythrocyte and reticulocyte 'ghosts' were inactive. 3. Liposomes prepared from pure lipids with net negative, positive or neutral charge did not possess inactivation capacity. 4. Liver plasma-membrane inactivation capacity was destroyed by heating at 50°C. 5. Inactivation factor solubilized from membranes by trypsin plus Triton X-100 treatment was partially purified by $(NH_4)_2SO_4$ fractionation, gel filtration, ion-exchange chromatography and hydroxyapatite chromatography. 6. Partially purified inactivation factor analysed by gel electrophoresis gave a major protein band that co-migrated with capacity for inactivation of glucose 6-phosphate dehydrogenase. 7. It is concluded that inactivation factor is a membrane protein whose intracellular distribution and other properties are consistent with a possible role for this activity in the initial step of protein degradation.

Inactivation has been suggested to play a crucial role in determining the relative rates of enzyme degradation *in vivo* (Hopgood & Ballard, 1974; Ballard & Hopgood, 1976; Goldberg & St. John, 1976). The evidence for this suggestion is mainly based on correlations between the stability of rat liver enzymes *in vitro* and their rates of degradation *in vivo* (Goldberg & Dice, 1974; Hopgood & Ballard, 1974; Bond, 1975, 1976). Additionally, several physical and chemical properties, including hydrophobicity, net charge, subunit size and proteolytic susceptibility, correlate with degradation rates *in vivo* (Goldberg & St. John, 1976). Most of these correlations can be explained in terms of protein conformation whereby proteins of short half-life would have unstable conformations or external bonds that are susceptible to proteinases or other factors that initiate proteolysis.

Selective denaturation on membrane surfaces has been proposed as one means by which protein degradation is initiated, with loss of catalytic activity an initial result when the proteins are enzymes (Dean, 1975; Bohley *et al.*, 1977; Ballard, 1977). Ballard & Hopgood (1976) described a membrane factor that was capable of inactivating rat liver cytosol enzymes at rates approximately proportional to their degradation rate constants measured in the intact animal (Hopgood & Ballard, 1974). In the present paper we extend the observations on intracellular distribution and describe the purification of this membrane factor. Part of the work described has been presented in preliminary form (Ballard *et al.*, 1977).

Materials and Methods

Chemicals

Substrates, enzymes, nucleotides required for the measurement of enzyme activities, lima-bean trypsin inhibitor, lipids and reagents for electrophoresis were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sepharose CL-6B was from Pharmacia (Uppsala, Sweden); hydroxyapatite gel was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Type DE-52 DEAE-cellulose was a product of Whatman Biochemicals (Maidstone, Kent, U.K.).

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Enzyme measurements

Assays for the following enzymes were carried out at the temperatures indicated without further modifications: phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32), 37°C (Ballard & Hanson, 1969); glucose 6-phosphatase (EC 3.1.3.9), 37°C (Shull *et al.*, 1956); 5'-nucleotidase (EC 3.1.3.5), 37°C (Aronson & Touster, 1974); acid phosphatase (EC 3.1.3.2), 37°C (Trouet, 1974); fructose 1,6-bisphosphatase (EC 3.1.3.11), 37°C (Clark *et al.*, 1973); fructose bisphosphate aldolase (EC 4.1.2.13), 25°C (Blostein & Rutter, 1963); lactate dehydrogenase (EC 1.1.1.27), 25°C (Kornberg, 1955); glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 25°C (Kornberg & Horecker, 1955). A unit of enzyme is that amount of activity that catalyses either the formation of 1 μmol of product or the removal of 1 μmol of substrate/min.

Standard inactivation assay

Livers from fed rats were homogenized in 3 vol. of 0.25 M-sucrose and centrifuged at 300000 *g* for 60 min. The clear supernatant was stored at −20°C for up to 2 months. Incubation mixtures contained 90 μl of this cytosol solution, 50 μl of freshly neutralized 10 mM-L-cystine, pH 8.0, 10 μl of 0.5 M-Tris/HCl buffer, pH 8.0, and a volume of sample to be assayed up to 100 μl containing Triton X-100 at 0.1%. Samples were taken after incubation at 37°C for various time periods and cooled in ice for subsequent measurement of cytosol enzyme activities. Inactivation activity was expressed as the percentage of initial activity remaining after a given time period or (where first-order kinetics are observed) the half-time for inactivation as determined by a semi-logarithmic plot. A unit of inactivation factor in the latter case was defined as the amount of activity that inactivates 1 unit of substrate enzyme/min at the inactivation half-time.

Partial purification of the inactivation factor

Preparation of microsomal fraction. Male rats of approx. 200–250 g body wt. were starved for 20 h, killed by a blow on the head and their livers rapidly added to 0.9% NaCl. Liver was minced with scissors and homogenized with 3 vol. of 0.25 M-sucrose in 5 mM-Tris/HCl buffer, pH 8, in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged for 200000 *g*-min and the pellet discarded. The supernatant was centrifuged for 6×10^6 *g*-min and the microsomal pellet resuspended in 50 mM-Tris/HCl buffer, pH 8, by homogenization in an all-glass Dounce homogenizer.

Extraction by tryptic digestion in the presence of Triton X-100. Microsomal preparations were incubated at 37°C for 90 min with mechanical stirring in 1% Triton X-100 containing 500 mg of trypsin for each 5 g of microsomal protein and buffered at pH 8. The digest was cooled to 0°C, centrifuged for 6×10^6 *g*-min and the supernatant retained.

Fractionation with $(NH_4)_2SO_4$. The digest supernatant was fractionated by slow addition of solid $(NH_4)_2SO_4$ to give a solution 40% saturated with respect to salt. The mixture was left for 60 min at 0°C before centrifuging for 30000 *g*-min. The supernatant was carefully siphoned away from the gelatinous floating pellet and then adjusted to 60% saturation with respect to $(NH_4)_2SO_4$. After standing and centrifugation as above, the pelleted material was dissolved in approx. 15 ml of 50 mM-Tris/HCl buffer, pH 8, containing 0.1% Triton X-100.

Gel filtration on Sepharose CL-6B. The $(NH_4)_2SO_4$ fraction was applied to a Sepharose CL-6B column equilibrated with 50 mM-Tris/HCl buffer, pH 8, and the inactivation factor was eluted with the same buffer.

Chromatography on DEAE-cellulose. The active fractions from gel filtration were pooled and pumped on to a DEAE-cellulose column (20 cm × 0.65 cm^2) equilibrated with 50 mM-Tris/HCl buffer, pH 8. Washing with the equilibration buffer, 50 mM-Tris/HCl buffer, pH 8, containing 0.1% Triton X-100, resulted in the elution of protein containing small amounts of inactivation capacity. However, the major peak was eluted by application of a 0–400 mM-NaCl gradient in the equilibration buffer. These latter fractions were pooled and diluted with an equal volume of 20 mM-KH_2PO_4 before being adjusted to pH 7 with 2 M-HCl.

Chromatography on hydroxyapatite. The eluate from DEAE-cellulose chromatography was pumped on to a column of hydroxyapatite as described in Fig. 2(*b*). The major peak of inactivation capacity was eluted by washing with about 5 column volumes of equilibration buffer. A second smaller peak of activity was eluted after the commencement of a 10–400 mM-KH_2PO_4, pH 7, gradient containing 0.1% Triton X-100. The active fractions were pooled, diluted to lower the salt concentration and passed through a DEAE-cellulose column (bed volume 1 ml) equilibrated with 50 mM-Tris/HCl buffer, pH 8, containing 0.1% Triton X-100. The inactivation factor was eluted in a small volume of the same buffer containing 0.5 M-NaCl.

Preparation of membranes

Rat cellular membranes were prepared by using the following methods: rough and smooth microsomal preparations (referred to below simply as 'microsomes') (Dallner, 1974); lysosomes (Trouet, 1974); erythrocyte and reticulocyte 'ghosts' (Steck, 1974); plasma membranes (Aronson & Touster, 1974); fat-cell 'ghosts' (Rodbell & Krishna, 1974).

To prepare liposomes, pure lipids (in chloroform/methanol, 2:1, v/v) were dried on the surface

of glass homogenizer under vacuum at 20°C. After homogenization with a tight plunger with 5mM-sodium phosphate buffer, pH 8, the suspension was sonicated for 10s at 5°C, centrifuged for $3 \times 10^6 g$-min and the opaque pellet resuspended by homogenization with a loose plunger. Liposomes prepared included cationic liposomes (20μmol of phosphatidylcholine, 20μmol of cholesterol and 10μmol of stearylamine), anionic liposomes (10μmol of phosphatidic acid in place of stearylamine) and neutral liposomes (20μmol of phosphatidylcholine and 20μmol of cholesterol). Freeze-dried microsomes were homogenized in a glass Dounce homogenizer with 10ml of butan-1-ol or chloroform/methanol (1:1, v/v), centrifuged at 50000g-min and the supernatant was used to prepare liposomes by the above method.

Phospholipase C digestion of microsomes

Microsomes (1mg of protein) were incubated with 6 units of *Clostridium welchii* phospholipase C at 37°C in the presence of 2.5mM-$CaCl_2$ at pH 8 for up to 4h. After addition of an equal volume of 10mM-EDTA, pH 6, to stop the reaction, the inactivation capacities of the treated microsomes were compared with those of controls (Zakim & Vessey, 1973).

Preparation of purified glucose 6-phosphate dehydrogenase

The enzyme used for inactivation studies had a specific activity of 79units/mg of protein and had been purified by the method of Matsuda & Yugari (1967). The enzyme preparation was stored at 0°C as a suspension in 3M-$(NH_4)_2SO_4$. Purified enzyme was centrifuged at 15000g for 10min and the pellet dissolved in 50mM-Tris/HCl buffer, pH 8, before use. The enzyme was judged to be pure from the single band of protein obtained by analytical sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis of purified inactivation factor was carried out by using 7.5% (w/v) acrylamide gels by the method of Dewald *et al.* (1974). Gels containing immobilized glucose 6-phosphate dehydrogenase were prepared for electrophoresis of inactivation factor at 2°C (Harrison, 1974). Inactivation factor was negatively stained by incubating the gels at 37°C for 1h to allow inactivation to proceed before staining for residual glucose 6-phosphate dehydrogenase activity (Harrison, 1974; Worsfold *et al.*, 1977). Protein was stained by using the method of Fazekas de St. Groth *et al.* (1963).

Protein measurements

Protein was determined by a modification (Dulley & Grieve, 1975) of the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as the standard.

Replication of experiments

The reported data in Tables and Figures are for individual experiments. Essentially similar results were obtained in repeats of every experiment.

Results and Discussion

Intracellular localization of the inactivation reaction

In a previous report it was shown that cytosolic enzymes such as phosphoenolpyruvate carboxykinase (GTP) and glucose 6-phosphate dehydrogenase were inactivated when homogenates from either fed or starved rats were incubated at 37°C in the presence of L-cystine (Hopgood & Ballard, 1974). Subsequent investigations showed that the factor inactivating phosphoenolpyruvate carboxykinase (GTP) co-sedimented with the microsomal marker glucose 6-phosphatase when a freeze-dried post-microsomal fraction was sedimented on a sucrose density gradient (Ballard & Hopgood, 1976). Although the activation capacity in this latter study was well separated from lysosomal and mitochondrial marker enzymes, it did not eliminate an involvement of membranes from these organelles, because the methods used would have lysed such structures. In an attempt to clarify this point, a number of different membrane fractions were prepared and their abilities to inactivate glucose 6-phosphate dehydrogenase tested (Table 1).

Highly purified lysosomes isolated from the livers of Triton WR-1339-treated rats possessed inactivation capacity. Further, lysosomal membranes prepared by alternate cycles of freezing and thawing in the presence of EDTA were active, even though this treatment removed most of the protein, half of the acid phosphatase, half of the β-*N*-acetylglucosaminidase and almost all of the β-galactosidase activity, in agreement with Baccino *et al.* (1971). Baccino *et al.* (1971) also showed removal of 87% of the acid proteinase activity from the particulate fraction by the freezing-and-thawing procedure. We note that the removal of lysosomal contents yielded membranes that when assayed at an alkaline pH were more active than a total lysosome preparation, making it unlikely that lysosomal acid proteinases were contributing significantly to the inactivation process. Plasma membranes derived from both the nuclear fraction (N_2 plasma membranes) and the microsomal fraction (P_2 plasma membranes) had the highest specific activity of any subcellular fraction tested. Substantial inactivation capacity was also present in smooth and rough microsomes, in agree-

Table 1. *Inactivation of glucose 6-phosphate dehydrogenase by various liver membranes*
Fractions tested (0.5 mg of protein/ml) were incubated with a cytosol fraction of rat liver in the presence of 2 mM-L-cystine at pH 8 and 37°C. Membranes were prepared and assayed as described in the Materials and Methods section to determine their capacity for inactivating glucose 6-phosphate dehydrogenase. 'Homogenate' represents total cellular particulate fractions added to cytosol in the inactivation assay. n.d., Not determined.

Source	Enzyme activity remaining after 1 h (%)	Relative enrichment of marker enzymes		
		5′-Nucleotidase	Glucose 6-phosphatase	Acid phosphatase
'Homogenate'	50	1.0	1.0	1.0
Smooth microsomes	18	3.1	1.2	0.6
Rough microsomes	17	3.0	2.8	1.6
Lysosomes	40	9.5	1.7	31
Lysosomal membranes	30	n.d.	n.d.	43
N_2 plasma membranes	5	22	1.1	1.4
P_2 plasma membranes	4	14	1.8	2.3
Hepatocyte P_2-plasma membranes	0	20	2.1	1.2

ment with previous experiments (Ballard & Hopgood, 1976). The distribution of the plasma-membrane marker enzyme 5′-nucleotidase suggests that plasma membranes are responsible for the activity associated with the lysosomal fraction. Thus preparations of Triton-filled lysosomes were always associated with a substantial enrichment of 5′-nucleotidase. Although the reliability of 5′-nucleotidase as the sole marker for plasma membranes in cell-fractionation experiments is complicated by membrane influx to form endocytic vacuoles and eventually lysosomes (Tulkens *et al.*, 1978), it appears reasonable to conclude that lysosomal membranes do not possess significant inactivation capacity. Perhaps these results are best interpreted as indicating a generalized membrane location for the factor with the greatest activity in the plasma membrane rather than a single membrane location with differential contamination in the various subcellular fractions.

To show that the liver plasma membranes inactivating glucose 6-phosphate dehydrogenase were derived from parenchymal cells rather than from endothelial cells, hepatocytes were isolated from collagenase-digested liver (Berry & Friend, 1969). These hepatic parenchymal cells were first purified by differential centrifugation and then used to prepare a plasma-membrane fraction. Such membranes were just as active as those prepared from whole liver and showed comparable enrichment of marker enzymes (Table 1).

A number of non-hepatic membranes were tested for their ability to inactivate glucose 6-phosphate dehydrogenase. Erythrocyte 'ghosts' are known to bind glyceraldehyde 3-phosphate dehydrogenase and aldolase from a number of different sources, leading to partial inactivation of these enzymes (Solti & Friedrich, 1976). However, erythrocyte 'ghosts' did not inactivate glucose 6-phosphate dehydrogenase in our system. Similarly reticulocytes, which carry out a wider range of metabolic activities, including the capability to display enhanced degradation of error proteins (Chandler & Ballard, 1978), yielded plasma membranes that lacked inactivation capacity. Of the non-hepatic cells tested, only the two that contain phosphoenolpyruvate carboxykinase (GTP) and glucose 6-phosphate dehydrogenase had measurable inactivation capacity. These are the Reuber H35 hepatoma, a cell line retaining several liver-specific functions (Pitot *et al.*, 1964), which possessed substantial activity in the plasma-membrane fraction, and adipocyte 'ghosts', which showed only a slight capacity to inactivate glucose 6-phosphate dehydrogenase.

A role for lipids rather than specific proteins in the inactivation system was investigated by attempting to destroy the capacity by phospholipase C treatment of microsomes. This was not successful. Also, liposomes prepared from microsomes by using either butan-1-ol or chloroform/methanol extraction displayed no activity. Likewise liposomes with net positive, negative or neutral charge made from purified lipids were ineffective.

During the initial studies problems were encountered when attempts were made to measure the inactivation activity of crude subcellular fractions, in that a variable lag period was observed and the reaction failed to show first-order kinetics. As a result only semi-quantitative results could be obtained, such as those given in Table 1. This problem has been largely overcome by performing the assay in the presence of 0.1% Triton X-100 under conditions where a maximum of 75% of the initial enzyme activity is lost during the assay period. Improved quantitative determination has aided attempts to purify this activity from a membrane source.

Partial purification of the inactivating factor

Studies on the subcellular distribution of the inactivating factor indicated the highest specific acti-

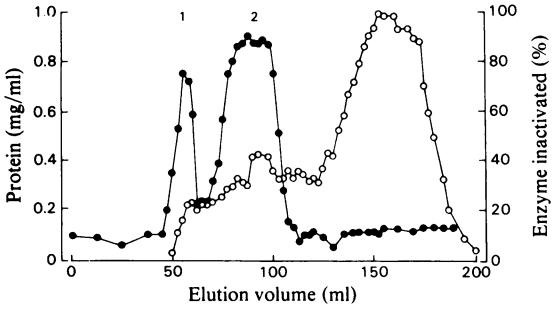

Fig. 1. *Separation of inactivation factor by Sephadex G-200 chromatography*

Washed liver microsomes (150mg of protein) were incubated at 37°C for 90min with 15mg of trypsin. Lima-bean trypsin inhibitor (7.5mg) was added and the mixtures were cooled in ice before being centrifuged at $9 \times 10^6 g$-min. The supernatant was concentrated by ultrafiltration, applied to a 90cm × 1.8cm² column of Sephadex G-200 equilibrated with 50mM-Tris/HCl buffer, pH 8, and eluted with the same buffer at a flow rate of 6ml/h. Portions of eluate were assayed for protein content (O) and for capacity for inactivation of glucose 6-phosphate dehydrogenase in the presence of trypsin inhibitor, and inactivation was expressed as a percentage of activity destroyed in 60min (●).

vity in plasma membranes, although crude microsomes contained much greater total activity. Accordingly microsomal membranes were used as the source for purification experiments. Attempts to solubilize the inactivation factor from a microsomal fraction by using high- or low-ionic-strength buffers were not successful, even with the aid of sonication (Ballard & Hopgood, 1976). Treatment of microsomes with 1% (w/v) Triton X-100 allowed recovery of a significant amount of the activity in a $6 \times 10^6 g$-min supernatant, but subsequent gel filtration on Sephadex G-100 or G-200 followed by ion-exchange chromatography failed to resolve the activity from the bulk of microsomal protein (Ballard & Hopgood, 1976). However, the digestion of membranes with trypsin resulted in the resolution of two peaks of activity on Sephadex G-200 chromatography (Fig. 1), one coincident with the protein breakthrough that presumably represents high-molecular-weight material (labelled 1 in Fig. 1), and a second that chromatographed within the gel matrix (labelled 2 in Fig. 1).

The extraction of factor from membranes with trypsin, Triton X-100 or trypsin plus Triton X-100 was compared in a subsequent $(NH_4)_2SO_4$ fractionation (Table 2). Digestion of microsomes with

Table 2. *Extraction of inactivation factor from microsomes*

Microsomes (60mg of protein) were extracted at 37°C at pH 8 for 90min by incubation with Triton X-100 (1% final concentration), trypsin (6mg) or trypsin plus Triton X-100 in a final volume of 6ml. The digest was cooled to 0°C and then centrifuged at $6 \times 10^6 g$-min, and 5ml of each supernatant was subjected to $(NH_4)_2SO_4$ fractionation as outlined in the text. The pellet from each step was dissolved in 2ml of 50mM-Tris/HCl buffer, pH 8, containing 1% Triton X-100. Portions of these fractions were assayed for capacity for inactivating glucose 6-phosphate dehydrogenase in the presence of lima-bean trypsin inhibitor.

Fraction	Extraction procedure ...	Enzyme activity remaining (% after 15min)		
		Trypsin	Triton X-100	Triton X-100 + trypsin
Extraction supernatant		100	62	78
0–30% $(NH_4)_2SO_4$ saturation		89	44	46
30–40% $(NH_4)_2SO_4$ saturation		87	69	81
40–50% $(NH_4)_2SO_4$ saturation		85	42	52
50–60% $(NH_4)_2SO_4$ saturation		83	81	50
60–70% $(NH_4)_2SO_4$ saturation		62	94	85
70–80% $(NH_4)_2SO_4$ saturation		79	94	90

Table 3. *Partial purification of inactivation capacity from microsomes*

Inactivation capacity was measured with glucose 6-phosphate dehydrogenase as substrate.

	Total protein (mg)	Total activity (units)	Specific activity (munits/mg of protein)	Purification (fold)	Yield (%)
Microsomes	5000	275	55	1.0	100
Extraction supernatant	4745	261	50	0.91	95
$(NH_4)_2SO_4$ pellet	950	52.3	55	1.0	19
Sepharose CL-6B	105	31.1	296	5.38	11
DEAE-cellulose, major peak	38	24.3	637	11.6	8.8
Hydroxyapatite (after concentration)					
Peak 1	1.7	10.8	6330	115	3.9
Peak 2	1.8	2.8	1530	27.8	1.0

trypsin in the presence of Triton X-100 gave a fraction that, when precipitated between 40 and 60% $(NH_4)_2SO_4$ saturation, was separated from higher-molecular-weight membrane aggregates on gel filtration (peak 2, Fig. 1). This was selected for further purification. Furthermore, the inactivation factor extracted in the presence of trypsin plus Triton X-100 was more easily purified than if detergent alone was used for extraction. Accordingly Triton X-100 plus trypsin was used in the subsequent purification experiments. All purification procedures were carried out at 4°C unless otherwise stated, and inactivation capacity was determined by using cytosol glucose 6-phosphate dehydrogenase in the presence of lima-bean trypsin inhibitor. The details of the purification scheme are described in the Materials and Methods section.

The purification is summarized in Table 3. Chromatography on Sepharose CL-6B and hydroxyapatite (Fig. 2) were the most effective steps in the purification procedure. Although DEAE-cellulose ion-exchange usually only gave 1–2-fold purification, its omission lowered the degree of purification on the subsequent hydroxyapatite column. The final material from six different purifications gave specific activities that varied over a 5-fold range. The relationship between the two peaks of activity from hydroxyapatite chromatography has not been estalbished, other than they both have isoelectric points between 6.0 and 6.25 as determined by isoelectric focusing on granulated slab gels by the method of Radola (1974).

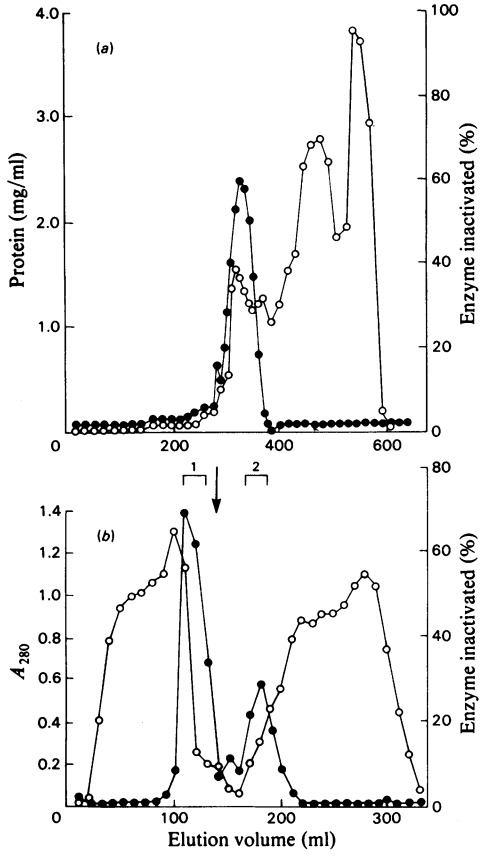

Fig. 2. *Gel filtration and hydroxyapatite chromatography of the inactivation factor*

(*a*) The $(NH_4)_2SO_4$ fraction was applied to a 90cm × 5cm² Sepharose CL-6B column equilibrated with 50mM-Tris/HCl buffer, pH 8, containing 0.1% Triton X-100, and eluted with the same buffer at a flow rate of 26ml/h. Fractions were assayed for protein content (○) and the amount of glucose 6-phosphate dehydrogenase inactivated in 15min (●). (*b*) Pooled fractions from DEAE-cellulose chromatography were pumped on to a 20cm × 0.65cm² hydroxyapatite column equilibrated with 10mM-potassium phosphate buffer, pH 7, containing 0.1% Triton X-100. After the column had been washed with equilibration buffer until the absorbance at 280nm (○) was below 0.2, a 10–400mM-phosphate gradient was applied (arrow). The amount of glucose 6-phosphate dehydrogenase inactivated in the standard assay was measured after 30min incubation (●). The two peaks of inactivation factor are indicated as 1 and 2.

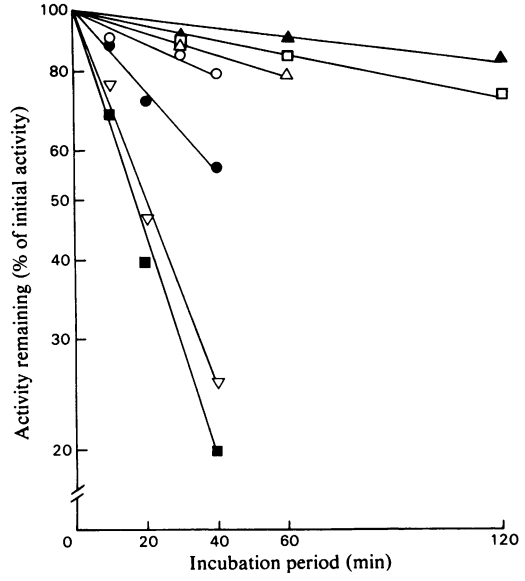

Fig. 3. *Assays of purified inactivation factor*

Purified factor (peak 1, Fig. 2*b*) was assayed as described in the Materials and Methods section. The amounts of factor used were 0.05μg (●), 0.1μg (□), 0.2μg (○), 0.4μg (△), 0.8μg (▲), 1.6μg (▽) and 2μg (■) of protein.

The inactivation of glucose 6-phosphate dehydrogenase by the purified factor approximately follows first-order kinetics (Fig. 3), in contrast with the complex inactivation curves reported previously for homogenates (Hopgood & Ballard, 1974) or membrane preparations (Fig. 4 below; see also Ballard & Hopgood, 1976). Further, the inactivation rate constant is approximately proportional to the concentration of purified factor over a 40-fold range (Fig. 3). This property greatly simplifies experiments on the mechanism of factor action.

Evidence that the inactivation factor is a membrane protein

Heating plasma membranes at 50°C or higher for 15 min destroyed the inactivation capacity, a result consistent with the factor being a protein (Fig. 4). This experiment incidentally demonstrates the non-linearity of the assay for inactivation factor when it is performed with unpurified preparations in the absence of Triton X-100. Additional evidence that the factor is a protein includes the failure to find a role for membrane lipids in the inactivation process and an increase of protein specific activity during the purification procedure.

Gel electrophoresis of the preparation described in Table 4 gave one major and several minor protein bands (Fig. 5). The major band of protein was coincident with capacity for inactivating glucose 6-phosphate dehydrogenase, as indicated by electrophoresis at 2°C on polyacrylamide gels containing immobilized glucose 6-phosphate dehydrogenase. This allowed negative staining for inactivation capacity (Fig. 5). Inactivation factor was not extracted from microsomes of plasma membranes by high- or low-ionic-strength buffers even when aided by sonication, but was solubilized by non-ionic detergent as high-molecular-weight aggregates. However, centri-

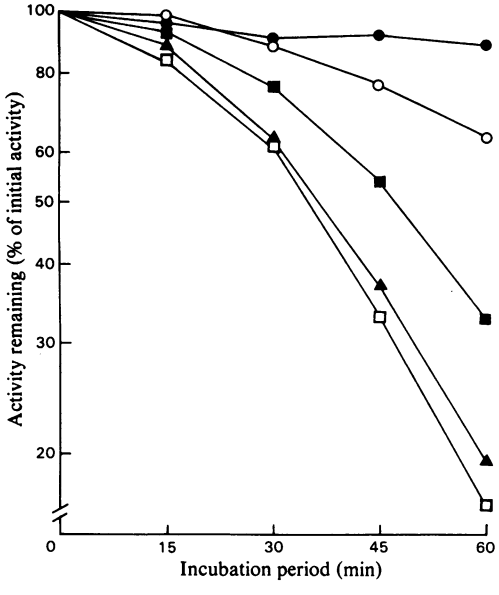

Fig. 4. *Heat inactivation of the plasma-membrane inactivation factor*

Plasma membranes from rat liver (70 μg of protein) were preincubated for 15 min in 50 mM-Tris/HCl buffer, pH 8, in a total volume of 100 μl at 37°C (●), 45°C (○), 50°C (■), 55°C (▲) or 100°C (□) before being cooled in ice. Liver cytosol (100 μl) and 10 mM-L-cystine, pH 8 (50 μl), were added and the mixtures were incubated for the indicated times at 37°C before being assayed for residual glucose 6-phosphate dehydrogenase activity.

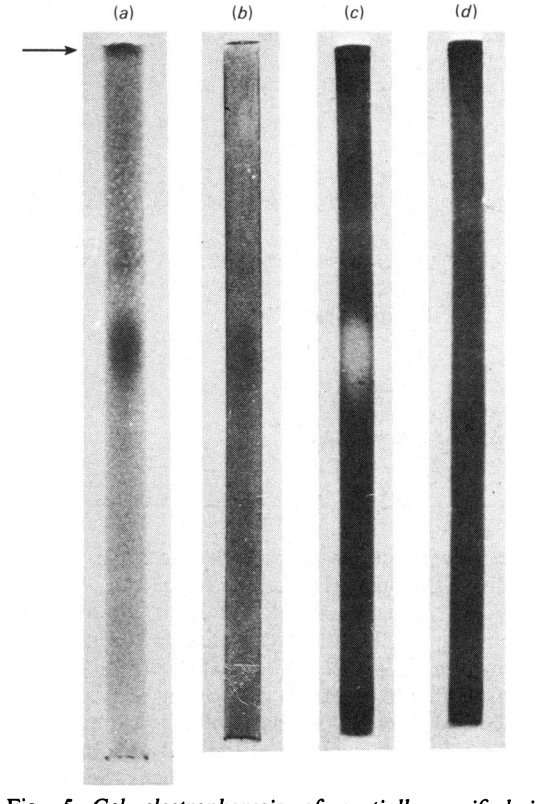

Fig. 5. *Gel electrophoresis of partially purified inactivation factor*

Polyacrylamide-gel electrophoresis at 2°C was performed as outlined in the Materials and Methods section. (*a*) Partially purified inactivation factor (25 μg of protein) was run on a standard gel and stained for protein. Simultaneous electrophoresis of partially purified factor on gels containing covalently immobilized glucose 6-phosphate dehydrogenase allowed staining of gels for both protein (*b*) and inactivation capacity (*c*). A control gel was similarly stained for inactivation capacity (*d*). The origin is indicated by an arrow.

Table 4. *Modification of membrane inactivation capacity*

Microsomes (0.46 mg of protein) were incubated at 37°C in the standard glucose 6-phosphate dehydrogenase inactivation assay together with various reagents at the indicated concentrations. Residual enzyme activity was measured at 20 min.

Additions	Enzyme activity remaining (% after 20 min)
None	100
Microsomes	22
Microsomes, Triton X-100 (10 g/l)	10
Microsomes, ethanol (40 ml/l)	0
Microsomes, urea (0.5 M)	3
Microsomes, dimethyl sulphoxide (200 ml/l)	73
Microsomes, ethylene glycol (200 ml/l)	61

Table 5. *Inactivation of liver cytosol enzymes by crude microsomes and the purified factor*

Microsomes (1.96 mg of protein) or purified factor (0.038 mg) were incubated at 37°C with 0.36 ml of rat liver cytosol in a solution of final volume 1.0 ml containing 0.5% Triton X-100 and 20 mM-Tris/HCl buffer, pH 8. Cystine (pH 8) was added where indicated at a concentration of 2 mM. Portions of the incubation mixtures were removed at various times for the determination of half-times of enzyme inactivation as described in the Materials and Methods section. Half-lives of the enzymes *in vivo* are from Hopgood & Ballard (1974) and Dice & Goldberg (1975).

Enzyme	Half-life *in vivo* (days)	Inactivation-factor source	Half-time for inactivation (min)	
			−Cystine	+Cystine
Phosphoenolpyruvate carboxykinase (GTP)	0.3	Microsomes	280	37
		Purified	81	20
Glucose 6-phosphate dehydrogenase	0.6	Microsomes	34	16
		Purified	28	14
Fructose 1,6-bisphosphatase	1.2	Microsomes	840	55
		Purified	460	45
Aldolase	4.9	Microsomes	Stable	60
		Purified	Stable	55
Lactate dehydrogenase	6.0	Microsomes	Stable	Stable
		Purified	Stable	Stable

fugation of such an extract at 175000 *g* pelleted increasing amounts of activity with time, approx. 30% after 1.5 h and 100% after 14 h. These observations suggest that the inactivation factor is an integral membrane protein. Consistent with this conclusion is the finding that inactivation capacity of a Triton X-100-detergent extract of microsomes was tightly bound on columns of the hydrophobic interaction chromatography medium phenyl-Sepharose CL-6B. Non-ionic detergents such as Triton X-100 and Brij 35, but not ethylene glycol, were able to elute the activity. Furthermore agents that perturb membrane structure (Lenaz, 1974) at concentrations that do not interfere with the stability of cytosolic glucose 6-phosphate dehydrogenase in the inactivation assay all stimulate the inactivation reaction. This response is found with Triton X-100, ethanol and urea (Table 4). On the other hand, compounds that are known to stabilize subcellular structure, such as ethylene glycol and dimethyl sulphoxide (Fleischer & Kervina, 1974), inhibit the rate of glucose 6-phosphate dehydrogenase inactivation (Table 4).

The main reason for purifying the inactivation factor was our observation showing a correlation for several enzymes between inactivation in homogenates and degradation *in vivo* (Hopgood & Ballard, 1974). The experiment in Table 5 shows that enzymes that are inactivated rapidly by microsomes are also inactivated by the purified factor. Similarly, aldolase and lactate dehydrogenase, which have long half-lives *in vivo*, are more slowly inactivated by both crude microsomes and the purified factor. Cystine stimulated the inactivation in all cases. We consider that, notwithstanding the low yield obtained during the purification of the inactivation factor, the final product is representative of a major part of the factor present in the starting homogenate.

The intracellular distribution and purification of the inactivation factor do little to resolve a role for the factor *in vivo*. However, protein degradation

appears to involve at least two pathways, one comprising the autophagolysosome system, which is regulated by hormones and nutrients, and a second, unregulated, pathway (Knowles & Ballard, 1976; Amenta *et al.*, 1977). We have proposed that the second pathway accounts for differences in degradation rate constants between proteins (Knowles & Ballard, 1976) and would thus be consistent with the properties of the membrane inactivation factor. Further experiments on the mechanism of the purified factor may resolve its importance, if any, in the intact cell.

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