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Specific inactivation of the phosphohydrolase component of the hepatic microsomal glucose-6-phosphatase system by diethyl pyrocarbonate

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We have examined the interactions of the histidine-specific reagent diethyl pyrocarbonate (DEPC) with the components of the rat hepatic glucose-6-phosphatase system (EC 3.1.3.9). DEPC is the first known reagent that satisfies the criteria of an active-site-specific label for the phosphohydrolase component. (*a*) It inactivates through formation of a stable covalent bond. (*b*) It is effective at reasonably low concentrations (2–4 mM) under relatively mild conditions (e.g. 30°C at neutral pH). (*c*) Inactivation is substantially blocked by glucose 6-phosphate, P_i and NaF, compounds which are known to interact quite specifically with the phosphohydrolase. (*d*) Under conditions where glucose 6-phosphate and NaF protect the enzyme, no protection is provided against DEPC-mediated inactivation of two other functional components of the membrane, the glucose 6-phosphate translocase and UDP-glucuronyltransferase. DEPC also shows potential for use at 0°C as a label for UDP-glucuronyltransferase.

Hepatic microsomal glucose-6-phosphatase (EC 3.1.3.9) is potentially the most important enzyme involved in the homoeostatic regulation of blood glucose concentrations (Ashmore & Weber, 1959). Substantial kinetic (Arion *et al.*, 1975, 1976*a*, 1980*b*) and genetic (Lange *et al.*, 1980; Nordlie *et al.*, 1983) evidence indicates that glucose 6-phosphate hydrolysis in the glucogenic tissues is catalysed by a multicomponent system. It has been proposed (Arion *et al.*, 1975) that the active site of glucose-6-phosphatase in intact microsomes (microsomal fractions) is situated at the lumenal surface of the membrane and that a specific translocase (T_1) mediates entry of glucose 6-phosphate.

Abbreviations used: DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid: [^{3}H]H_2DIDS, ^{3}H-labelled 4,4'-di-isothiocyano-1,2-diphenylethane-2,2'-disulphonic acid; DEPC, diethyl pyrocarbonate (ethoxyformic anhydride); Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; T_1, a glucose 6-phosphate-specific translocase that mediates the penetration of the hexose phosphate into the cisternae of the endoplasmic reticulum; T_2, a phosphate translocase that mediates efflux of P_i; k or $k_{inactivation}$, a first-order rate constant for inactivation of the glucose-6-phosphate phosphohydrolase.

P_i released at the lumenal surface is believed to equilibrate via a second translocase, T_2 (Arion *et al.*, 1980*b*).

The molecular basis of the multicomponent glucose-6-phosphatase system has been examined in several laboratories. A polypeptide (M_r 54000) specifically involved in the transport of glucose 6-phosphate has been identified by chemical labelling with [^{3}H]H_2DIDS (Zoccoli & Karnovsky, 1980; Zoccoli *et al.*, 1982). Partial purification and immunochemical studies have tentatively identified a small polypeptide ($M_r \simeq 20000$) to be a component of glucose-6-phosphatase (Burchell & Burchell, 1982; A. Burchell & W. J. Arion, unpublished work). We have searched for a specific chemical label for the glucose-6-phosphatase to confirm its identification and facilitate quantification of the phosphohydrolase component.

Earlier studies suggested a histidine residue at the active site of the phosphohydrolase (Nordlie & Lygre, 1966) which may become phosphorylated as an intermediate of the catalytic mechanism (Feldman & Butler, 1969). Thus we have used the chemical reagent DEPC, which is known to react with histidine residues in various proteins (see

Miles, 1977). The results described herein show that DEPC can be used to inactivate glucose-6-phosphatase specifically. DEPC is the first such agent to be identified, and thus it shows promise as a means to label selectively the active site of the enzyme. A preliminary report describing part of these studies has been presented (Arion *et al.*, 1984).

Materials and methods

Definitions

In this paper, microsomes isolated from liver homogenates, washed and assayed without further treatment are referred to as 'untreated'. As noted previously (Arion *et al.*, 1972*b*, 1976*a*, 1980*a*,*b*), untreated microsomes are mixtures of intact vesicles ('intact microsomes'), in which the limiting membrane acts as a selective permeability barrier, and disrupted structures in which selective permeability is lacking, so the enzyme has free access to ionic substrates and inhibitors. The proportion of the two forms is easily quantified by assays of the 'low-K_m' mannose-6-phosphatase activity that is expressed only in disrupted structures (Arion *et al.*, 1976*a*; Lange *et al.*, 1980; Arion & Walls, 1982). The enzymic activity of intact microsomes is calculated as the activity of untreated microsomes minus the contribution of enzyme in the disrupted component (Arion *et al.*, 1980*a*,*b*; Lange *et al.*, 1980; Arion & Walls, 1982). Untreated microsomes are converted into fully disrupted microsomes by exposure to detergents or NH_3, which completely destroys the selective permeability of the membrane (Arion *et al.*, 1972*a*,*b*, 1975, 1976*b*; Nilsson *et al.*, 1978).

Enzymic activities are expressed as units (or munits) per mg of protein, where a unit is the amount of enzyme that transforms 1 μmol of substrate to product in 1 min.

Materials

Glucose 6-phosphate (monosodium salt), mannose 6-phosphate (disodium salt), sodium cholate and DEPC were obtained from Sigma (London) Chemical Co. and used without further purification. Sodium cacodylate, also from Sigma, was recrystallized from 95% ethanol (Arion & Wallin, 1973). NaF, Hepes and imidazole were purchased from BDH Chemicals, Poole, Dorset, U.K. Stock solutions of DEPC were prepared in ethanol, which was maintained anhydrous by storage at ambient temperature over Sieve 4A (BDH Chemicals).

Enzyme preparations and assays

Male Wistar rats (approx. 200g) were starved for 24h and hepatic microsomes were prepared as described by Arion *et al.* (1980*a*), except that 5mM-Hepes, pH7.4, replaced Tris/acetate in the media used for homogenization, washing and storage of microsomes. Fully disrupted microsomes were prepared from untreated microsomes either by exposure at pH7.5 to 0.5% (w/v) sodium cholate for 60min at 0°C (Arion *et al.*, 1972*b*; Carlson, 1973) or by treatment with 0.1M-NH_3 (Stetten & Burnett, 1967) as described previously (Arion *et al.*, 1976*b*). Untreated microsomes and NH_3-disrupted microsomes were suspended in 0.25M-sucrose/5mM-Hepes, pH7.4, and stored at −70°C until used, when they were thawed at 0°C. The intactness of microsomal vesicles was assessed by determining the latency of mannose-6-phosphatase (Arion *et al.*, 1976*a*).

Glucose-6-phosphatase and mannose-6-phosphatase activities were assayed at pH6.5 by a micro-volume modification of the procedure described by Bickerstaff & Burchell (1980). Corrections were made for the contribution to glucose 6-phosphate hydrolysis by the 'disrupted component' in untreated microsomes as described previously (Lange *et al.*, 1980; Arion *et al.*, 1980*a*; Arion & Walls, 1982). 1-Naphthol-UDP-glucuronyltransferase (EC 2.4.1.17) was assayed radiochemically at pH7.4 (Otani *et al.*, 1976). Rates of glucuronidation in untreated microsomes were measured in both the absence (i.e. 'basal rates') and the presence of 4mM-UDP-*N*-acetylglucosamine, the putative physiological regulator of glucuronidation (see Dutton, 1980). The mean values from at least two determinations of enzyme activity are reported in all cases. Protein concentration was determined by a modification (Markwell *et al.*, 1978) of the procedure of Lowry *et al.* (1951), with crystalline bovine serum albumin as the reference standard.

Incubation of microsomal preparations with DEPC

NH_3-disrupted microsomes (1mg of protein) or untreated microsomes (2mg of protein) were incubated at either 0°C or 30°C in a final volume of 1ml in media, pH6.5, containing 20mM-sodium cacodylate, 1% (v/v) ethanol, and varied concentrations of DEPC, glucose 6-phosphate, NaF or other potential 'protective agents'. All reagents except DEPC and microsomes were equilibrated at the desired temperature, and microsomes were added at timed intervals. The reaction with DEPC was initiated 1min later by addition of 10μl of DEPC solution. Control incubations containing 10μl of ethanol were run concurrently. At the desired time, the reaction was terminated by addition of 1ml of ice-cold 0.1M-imidazole, pH7.5 (Miles, 1977). The mixtures were underlayered with 2.0ml of 0.5M-sucrose/10mM-Hepes, pH7.4, and the microsomes quantitatively recovered separate from protective agents and conjugated DEPC by sedimentation at 105000*g* at 4°C in a fixed-angle rotor (e.g. Beckman 50Ti or MSE 10 × 10ml Al). The microsomes

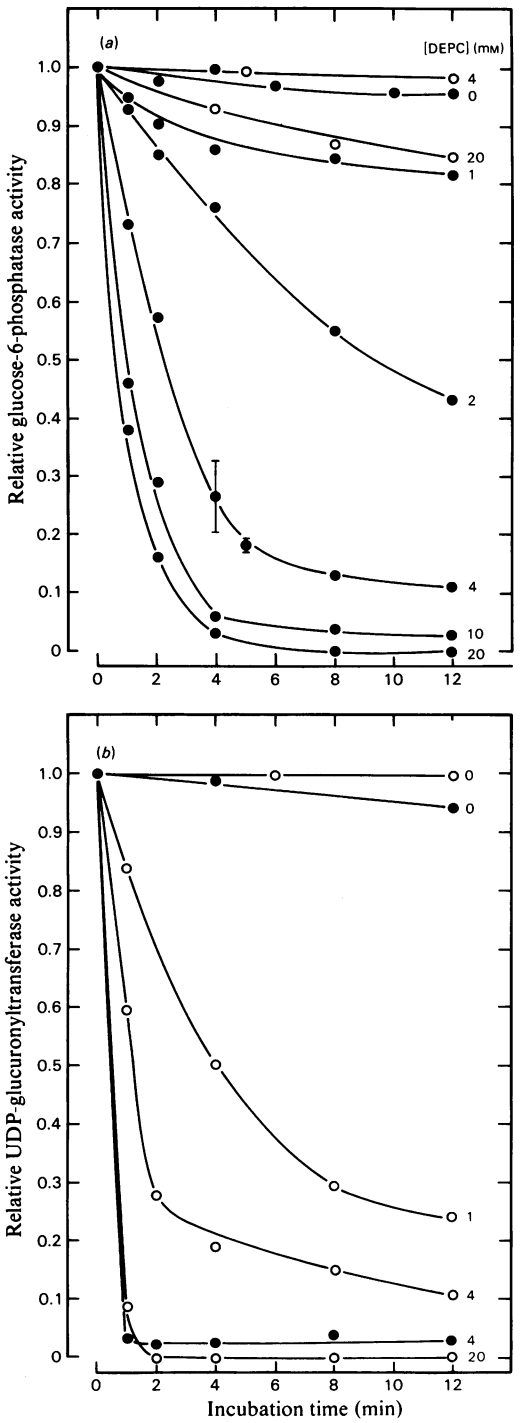

Fig. 1. *Influence of DEPC concentration and incubation temperature on the kinetics of inactivation of glucose-6-phosphatase (a) and 1-naphthol UDP-glucuronyltransferase (b)*

○, Treatment with DEPC at 0°C; ●, treatment with DEPC at 30°C. All values are compared with results of control incubations at 0°C in the presence of ethanol. Other details are given in the Materials and methods section.

were resuspended in 1ml of 0.25M-sucrose/5mM-Hepes, pH7.4, treated with cholate if desired, and appropriately diluted with suspending medium so that the addition of 20μl samples to the phosphohydrolase assay media (80μl) would yield initial rates of hydrolysis under all circumstances.

For the second exposure to DEPC, 50μl of 0.2M-Hepes, pH7.4, was added to 0.4ml of microsomes from the initial treatment. Portions (0.4ml) of these mixtures were brought to treatment temperature by incubation at 30°C for exactly 1min or at 20°C for 4min, and supplemented with 4μl of either 0.4M-DEPC or ethanol. At 1min intervals, 50μl samples were transferred to tubes containing 50μl of ice-cold 0.1M-imidazole, pH7.5, mixed rapidly and kept on ice until enzyme assays were performed.

Results

Conditions for inactivation of glucose-6-phosphatase by DEPC

In preliminary studies, untreated or NH_3-disrupted microsomes were incubated at 0°C with 1mM-DEPC for up to 30min. When assayed at 30mM-glucose 6-phosphate, 70% of the glucose-6-phosphatase activity of untreated microsomes was inactivated by exposure to DEPC for 10min, whereas the glucose-6-phosphatase activity of NH_3-disrupted membranes was unaffected even after 30min (results not shown). These responses are similar to those observed when intact microsomes were exposed to certain reagents which react with thiol-containing residues (Wallin & Arion, 1972), diazobenzenesulphonate (Nilsson *et al.*, 1978) or DIDS (Zoccoli & Karnovsky, 1980). They suggest (see Arion *et al.*, 1980*b*) that DEPC reacted at a site on the glucose 6-phosphate translocase in intact membranes and blocked access of the enzyme to glucose 6-phosphate, but that even in the absence of a potential barrier to its access to the hydrolytic site (i.e. the intact microsomal membrane) at 0°C the reagent did not form a covalent bond with a group at the catalytic centre.

The influences of DEPC concentration and treatment temperature on the kinetics of inactivation of glucose-6-phosphatase in NH_3-disrupted microsomes are shown in Fig. 1(*a*). The data show that incubation temperature is a critical factor in the inactivation of enzyme by DEPC. When 20mM-DEPC was used at 0°C (Fig. 1*a*) only 12% inactivation was seen after 8min, whereas at 30°C the enzyme was completely inactivated. Insignifi-

cant losses of activity were observed at either temperature in control incubations containing 1% ethanol.

Exposure of microsomes to 4mM-DEPC at 30°C for 4 or 5min resulted in approx. 70% and 80% inactivation respectively. The inactivation reaction was quite reproducible under these conditions. The error bars in Fig. 1(*a*) define the range of values observed in five independent experiments at the two time points. Moreover, plots of the natural logarithm of activity against time of exposure showed that inactivation was a first-order reaction under these treatment conditions (plots not shown). Consequently, these conditions were chosen for further evaluation of the inactivation reaction.

Fig. 1(*b*) illustrates that exposure of NH_3-disrupted microsomes to DEPC also caused inactivation of a second microsomal enzyme, UDP-glucuronyltransferase, although with sensitivity markedly different from that observed for the glucose-6-phosphatase. Glucuronidation of 1-naphthol was completely abolished within 2min by incubation with 20mM-DEPC at 0°C, a condition that inactivated only 5% of the glucose-6-phosphatase activity (cf. Fig. 1*a*). Whereas incubation for 4min at 0°C with 4mM-DEPC inactivated over 75% of the UDP-glucuronyltransferase activity, this condition had essentially no effect on glucose-6-phosphatase activity.

Protection against inactivation of glucose-6-phosphatase by glucose 6-phosphate and NaF

We examined the possibility of selectively blocking DEPC inactivation of the enzyme with substrate (glucose 6-phosphate), NaF, a reagent which stabilizes the glucose-6-phosphatase activity (Burchell & Burchell, 1980), and P_i, a hydrolytic product which is a competitive inhibitor of the hydrolytic reaction (Arion *et al.*, 1980*b*). The concentration-dependence for protection by these compounds is shown in Fig. 2. The inactivation of glucose-6-phosphatase at 30°C by 4mM-DEPC (84% after 5min incubation) was decreased to 16% in the presence of 25mM-NaF. Similarly, 10–60mM-glucose 6-phosphate or 30mM-P_i provided substantial protection against inactivation observed after 4min exposure. In contrast, the other hydrolytic product, D-glucose, which is a non-competitive inhibitor of the hydrolytic reaction (Arion & Wallin, 1973), provided only modest protection when present at 60mM. The protection afforded by NaF, glucose 6-phosphate or P_i was not the result of their direct reaction with DEPC, since in control incubations without microsomes none of the protective agents decreased the amount of DEPC available to react at pH7.5 with 10mM-imidazole (Miles, 1977). It should be noted that, despite an apparent 'saturation response', neither NaF or glucose 6-phosphate was able to provide complete protection against the action of DEPC.

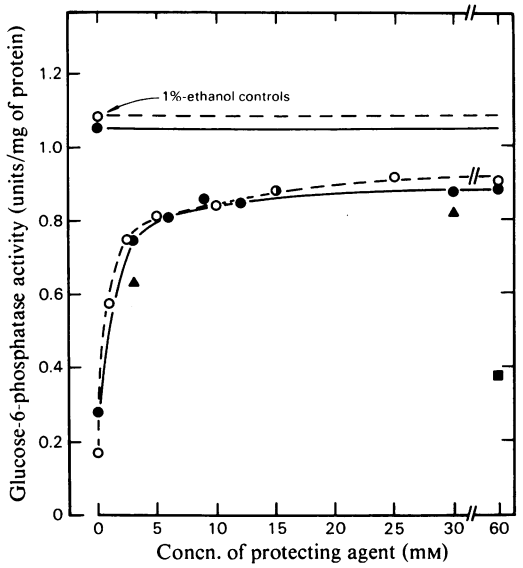

Fig. 2. *Concentration-dependence of protection against DEPC-induced inactivation by NaF, glucose 6-phosphate and P_i*

NH_3-disrupted microsomes (1 mg/ml) were exposed to 4mM-DEPC at 30°C and pH6.5 for 5min when NaF was used as the protecting agent and in all other cases for 4min under the same conditions: ▲, P_i; ■, D-glucose; ○, NaF; ●, glucose 6-phosphate.

The selectivity of the protection by glucose 6-phosphate and P_i is supported by the agreement between the concentrations of P_i and glucose 6-phosphate which provided 'half-maximal' protection and the values for the dissociation constants previously determined at pH6.5 for the interactions of the enzyme in fully disrupted microsomes with P_i (K_i = 3mM; Arion *et al.*, 1980*b*) and glucose 6-phosphate (K_s = 1.3mM; Arion & Wallin, 1973). The conclusion that half-maximal protection was elicited by 3mM-P_i is directly discernible by simple inspection of Fig. 2. More specifically, the addition of 3mM-P_i decreased the first-order rate constant for inactivation by DEPC ($k_{inactivation}$) from 0.33 to 0.15min^{-1}. The influence of added glucose 6-phosphate and NaF on $k_{inactivation}$ is shown in Fig. 3. It can be calculated from the equations defining the plots that 0.8mM-glucose 6-phosphate or 0.4mM-NaF caused $k_{inactivation}$ to be halved.

The selectivity of the protection afforded by glucose 6-phosphate and NaF against DEPC inactivation of the phosphohydrolase was subjected to further scrutiny in the experiment summarized in Table 1. Untreated microsomes (initially 95% intact) were exposed for 5min at 30°C to 4mM-

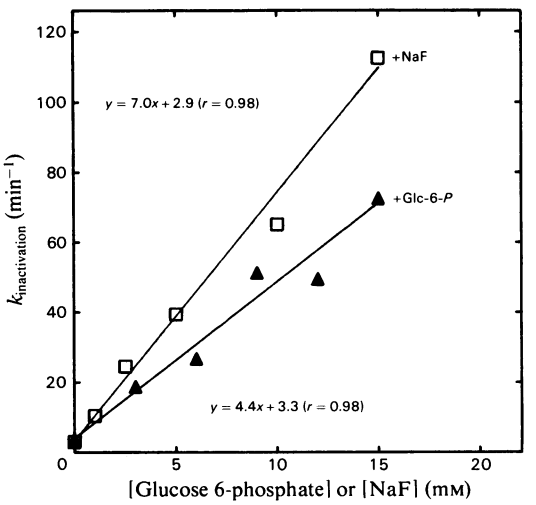

Fig. 3. *Influence of NaF and glucose 6-phosphate on the apparent first-order rate constant for DEPC-induced inactivation*

Estimates of $k_{inactivation}$ (min^{-1}) were calculated from the data in Fig. 2 by using the formula:

$k_{inactivation} =$
$(\ln A_0 - \ln A)$/time (min) of exposure to DEPC

where A_0 is the enzymic activity in fully protected preparations and A is activity after DEPC treatment.

Table 1. *Inactivation of glucose-6-phosphatase in intact microsomes by DEPC at 30°C*

Untreated rat liver microsomes (2mg of protein/ml) were exposed at pH6.5 to 4mM-DEPC for 5min at 30°C in the presence of the indicated additions. After isolation, the microsomes were assayed before and after supplementation to 0.5% sodium cholate. Other details are given in the Materials and methods section. Phosphohydrolase activities were assayed at 30°C in media, pH6.5, containing 30mM-glucose 6-phosphate or 1mM-mannose 6-phosphate and 10μg of untreated microsomes or 4μg of cholate-disrupted microsomes. Activity values for intact microsomes were normalized to correspond to that expected if all the enzyme in fully disrupted microsomes was housed in intact vesicles (Arion *et al.*, 1980*a*,*b*). Values in parentheses are the percentage inactivation relative to the 'ethanol control'.

Additions to treatment media	Glucose-6-phosphatase activity (unit/mg of protein)	
	Intact microsomes	Disrupted microsomes
A. 1% Ethanol (control)	0.38 (0)	0.51 (0)
B. 4mM-DEPC	0.070 (82)	0.27 (47)
C. 4mM-DEPC + 50mM-NaF	0.062 (84)	0.36 (29)
D. 4mM-DEPC + 150mM-glucose 6-phosphate	0.026 (93)	0.48 (6)

DEPC in the presence of the indicated additions. After isolation, glucose-6-phosphatase was assayed before and after disruption of the membranes with 0.5% cholate to permit independent assessments of the effects of treatment on the coupled system and the hydrolytic site, respectively. Glucose-6-phosphatase activity in intact microsomes was more than 80% inhibited by treatment with DEPC, whereas the phosphohydrolase activity was decreased by about 50% after cholate disruption of DEPC-treated microsomes. These responses, like those noted above for treatment at 0°C, indicate that DEPC preferentially inactivated the glucose 6-phosphate transporter. More significant, however, was the finding that, under the conditions used, where the hydrolytic site can be substantially protected (cf. Fig. 2), the presence of relatively high concentrations of NaF and glucose 6-phosphate afforded no protection to the translocase.

The experiment summarized in Table 2 shows that both the basal rate of 1-naphthol glucuronidation and that stimulated by UDP-*N*-acetylglucosamine were largely inactivated when untreated microsomes were exposed to DEPC at 30°C, but neither activity was protected by the presence of glucose 6-phosphate or NaF.

In a third experiment (results not shown), NH_3-disrupted microsomes were exposed to the same treatments described in Table 1. This treatment caused a 70% loss in the glucose-6-phosphatase activity (assayed at 30mM-glucose 6-phosphate), which was decreased to only 17 and 15% when treatment was performed in the presence of 50mM-NaF and 150mM-glucose 6-phosphate respec-

Table 2. *Absence of protection by NaF or glucose 6-phosphate during DEPC-induced inactivation of 1-naphthol UDP-glucuronyltransferase at 30°C*

The microsomes obtained from the treatments described in Table 1 were assayed before cholate supplementation for UDP-glucuronyltransferase activity in media lacking (i.e. 'basal' activity) or containing 4mM-UDP-*N*-acetylglucosamine. Other details are given in the Materials and methods section. Values in parentheses are the percentage inactivation relative to the 'ethanol control'.

Additions to treatment media	UDP-glucuronyltransferase activity (munits/mg of protein)	
	'Basal'	+UDP-*N*-acetylglucosamine
A. 1% Ethanol (control)	6.4 (0)	15 (0)
B. 4mM-DEPC	1.7 (73)	1.5 (90)
C. 4mM-DEPC + 50mM-NaF	1.3 (80)	1.0 (93)
D. 4mM-DEPC + 150mM-glucose 6-phosphate	0.1 (98)	1.6 (89)

tively. In contrast, 1-naphthol glucuronidation (initially 65 munits/mg of protein) was decreased to 3% of the control value after exposure to DEPC, and no protection was afforded by the presence of either 50 mM-NaF or 150 mM-glucose 6-phosphate.

Validation of a protocol for selective labelling of glucose-6-phosphatase

In the experiment shown in Fig. 4, NH_3-disrupted microsomes were subjected to a sequence of two exposures to 4 mM-DEPC, the first at pH 6.5 in the presence of 25 mM-NaF or 60 mM-glucose 6-phosphate and the second at pH 7.5 in the absence of protecting agent. The results confirm that substrate and NaF confer substantial protection during the first exposure and in addition demonstrate the critical feature that the protected activity can be inactivated by a second exposure to DEPC. The first-order rate constants for inactivation during the initial treatment ($k = 0.34 min^{-1}$) and second exposures ($k = 0.36$ and $0.34 min^{-1}$ for preparations protected by NaF and glucose 6-phosphate respectively) did not differ significantly, nor was the kinetics of inactivation during the second exposure noticeably influenced by the choice of protecting agent used during the initial exposure. Virtually identical results were obtained in other experiments (results not shown) when either 30 mM-P_i or the combination of 25 mM-NaF and 60 mM-glucose 6-phosphate was used to protect activity during the first exposure to DEPC.

The data in Fig. 4 show that the first exposure to DEPC in the presence of protecting agents caused the enzyme to become unstable when subsequently incubated at 30°C in the absence of DEPC. In contrast, the residual activity which survived the first exposure to DEPC in the absence of protecting agent was as stable during a second exposure at 30°C as the enzyme in control microsomes (see line denoted by ▲--▲ in Fig. 4). Supplementary studies showed that the presence of 1% ethanol was not an important factor in the thermal instability noted during the second exposure.

The inactivation observed during the second exposure at 30°C in the absence of DEPC also appeared to be a first-order process. It was consistently found, as illustrated in Fig. 1, that when glucose 6-phosphate was used as the protecting agent during the first exposure to DEPC the inactivation elicited by the subsequent heating in the absence of DEPC ($k = 0.06 min^{-1}$) was significantly greater than when protection during the first step was provided by NaF ($k = 0.03 min^{-1}$) or 30 mM-P_i (results not shown; $k = 0.03 min^{-1}$). Furthermore, the greater instability induced by the initial exposure to DEPC and glucose 6-phosphate was not affected by the presence of 25 mM-NaF; however, inactivation during the second incubation at 30°C was largely abolished if 25 mM-NaF was present (results not shown).

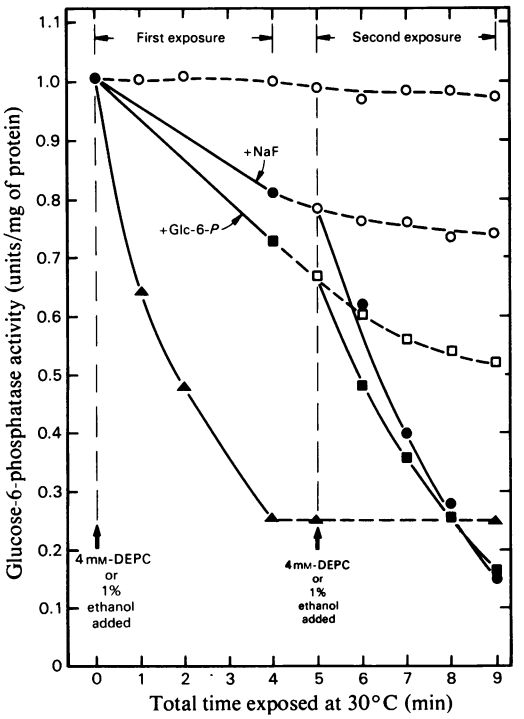

Fig. 4. *Validation of a protocol for selective 'labelling' of the catalytic centre of glucose-6-phosphatase with DEPC*
Data points (open symbols) for control incubations containing 1% ethanol are connected by broken lines. DEPC-induced inactivation in all cases is indicated by continuous lines. The protecting agent added to the first incubation with DEPC is denoted by ▲ (none), ■ (60 mM-glucose 6-phosphate) and ● (25 mM-NaF). Other details are given in the Materials and methods section.

Discussion

The present study is preliminary to a long-term goal of selectively labelling hepatic microsomal glucose-6-phosphatase so that the identity of the enzyme, which has eluded conventional approaches to purification (see Burchell & Burchell, 1982), can be unequivocally established. An active-site-specific label also would permit quantification of the phosphohydrolase component. DEPC appears to be the only chemical agent thus far identified which satisfies the requisite criteria for site-specific labelling of microsomal glucose-6-phosphatase. It is a covalently bound inhibitor that is effective at reasonably low concentations (e.g. 2–4 mM) under relatively mild conditions (i.e. 30°C and neutral pH). Inactivation is effectively blocked by compounds known to interact more or

less specifically with the phosphohydrolase: its substrate, glucose 6-phosphate, a competitive inhibitor of the phosphohydrolase activity, P_i (Arion *et al.*, 1980*b*), and NaF, an unusually effective stabilizer of the enzyme (Burchell & Burchell, 1980, 1982). Under identical conditions glucose 6-phosphate and NaF were unable to prevent inactivation of two other functional components of the membrane, the glucose 6-phosphate translocase and UDP-glucuronyltransferase.

We interpret the results of the experiment summarized in Fig. 4 as validating a protocol for selective labelling of the active site of glucose-6-phosphatase. Although the instability caused by the first exposure to DEPC in the presence of protecting agent creates less than an ideal situation, it is not expected to pose a serious obstacle to adequate labelling of the enzyme, since over 50% (when protected by glucose 6-phosphate) and over 70% (when protected by NaF) of the total inactivation observed during both exposures can be attributed to the reaction with DEPC during the second exposure.

The molecular basis for the instability induced by exposures to DEPC in the presence of protecting agents, especially glucose 6-phosphate, is obscure. We have, however, explored conditions for the second exposure to minimize inactivation unrelated to the direct reaction with DEPC. The decision to maintain pH at 7.4 rather than 6.5 during the second exposure derived from the earlier finding that lability of the enzyme when exposed at 30°C increases as the pH is decreased (Arion *et al.*, 1976*b*). The increase in pH had no discernible effect on the first-order rate constant for DEPC-dependent inactivation. We also found that inactivation during the second exposure could be significantly decreased by decreasing the incubation temperature to 20°C. However, $k_{inactivation}$ for DEPC at 20°C (approx. $0.08\,min^{-1}$) was less than one-quarter of the value determined at 30°C. Therefore, what can be gained in decreased lability is largely offset by the longer exposure times (e.g. 15 min) that would be needed to achieve significant labelling (e.g. 70%) of the enzyme at 20°C.

On a molar basis, NaF is somewhat better than glucose 6-phosphate in blocking inactivation by DEPC (Fig. 3), and whereas 1 mM-NaF gave substantial protection (Fig. 2), additional studies showed that NaCl was completely ineffective even when present at 25 mM. Although the molecular basis for the protection against DEPC inactivation provided by glucose 6-phosphate and the competitive inhibitor, P_i, is readily understandable, the reason why NaF is so effective is not so obvious. Little is presently known concerning the interactions of the phosphohydrolase with NaF, other than that the latter is one of the most effective agents for stabilizing the enzyme during attempts to purify it (Burchell & Burchell, 1980, 1982), and it has been reported to be an inhibitor of glucose 6-phosphate hydrolysis (Beaufay *et al.*, 1954).

The results in Table 1 show that the phosphohydrolase component of the glucose-6-phosphatase system, which is located on the lumenal surface of the membrane (Arion *et al.*, 1975; Nilsson *et al.*, 1978), can be inactivated by exposure of intact microsomes to DEPC. This observation indicates that the small (M_r 162) uncharged DEPC molecules can penetrate the microsomal permeability barrier, perhaps via the same mechanism that supports simple diffusion of D-glucose and other non-electrolytes smaller than M_r 1000 (Nilsson *et al.*, 1973; Ballas & Arion, 1978; Arion *et al.*, 1980*b*). Some degree of penetration of NaF into the cisternae also is indicated by the partial protection of enzyme in intact microsomes by NaF.

Our data suggest that DEPC treatment at 0°C may have potential to achieve site-specific labelling of UDP-glucuronyltransferase, but this remains to be validated. Other results (W. J. Arion & J. L. Countaway, unpublished work) indicate that DEPC has less promise as a site-directed agent for labelling the transport components of the glucose-6-phosphatase system. Although treatment at 0°C with 1 mM- or 2 mM-DEPC will effectively inhibit glucose 6-phosphate hydrolysis by intact microsomes without altering the enzyme, under all conditions that we have tested, including treatments which caused only modest inactivation, glucose 6-phosphate up to 0.2 M was totally ineffective in preventing inactivation.

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