ferase A; conjugations with epoxides such as styrene-7,8-oxide (STOX) are for the greater part catalyzed by transferase E[4].

Inhibition studies were performed with rat liver cytosolic GSH S-transferases using CDNB and STOX as substrates, and dBSP and tBSP as inhibitors. dBSP was donated by Dr D.K.F. Meijer (University of Groningen, The Netherlands), tBSP was obtained from Aldrich Chemical Co. (Milwaukee, USA). Radiolabelled STOX (7-^{3}H) with a sp. act. of 33 mCi/mmole was produced by The Radiochemical Centre (Amersham, U.K.). The enzymic conjugation of GSH with CDNB was assayed as reported before,[5] the conjugation with labelled STOX was measured according to Marniemi and Parkki[6]. Rat (♂) liver 105,000 × g supernatant served as the source of enzyme activities. In both assays the GSH concentration was fixed at 0.5 mM. The addition of inhibitor was followed by a preincubation period of 2 min after which the reaction was started by addition of the electrophilic substrate.

The figure shows experiments in which GSH S-transferase activity towards STOX or CDNB was inhibited by either dBSP or tBSP. Linear Lineweaver-Burk plots were obtained, indicating that the rate of the enzymatically catalyzed conjugations can be described by the Michaelis-Menten equation. The results show a non-competitive type of inhibition of transferase activity with CDNB as the substrate (apparent K_i of tBSP: 0.03 mM, apparent K_i of dBSP: 0.24 mM), although tBSP and CDNB are both good substrates for transferase A and show little (if any) enzymatic activity with the other transferases.[4] Obviously, the type of inhibition of dBSP is comparable with that of tBSP, albeit with an 8-fold greater apparent K_i. Surprisingly, applying STOX as the substrate results in a competetive type of inhibition with dBSP (apparent K_i: 0.19 mM) and a non-competetive type of inhibition with tBSP (apparent K_i: 0.03 mM), although tBSP has little or no activity towards transferase E, and dBSP is not a substrate at all.[4]

As, in vivo, tBSP is partly biotransformed to its GSH-conjugate, while dBSP is not metabolized[7], these compounds seem to be useful tools for the study of GSH S-transferases and the involvement of this enzyme system in the disposition of xenobiotics in vivo as well as in vitro. It should be noted however that GSH S-transferase B has been identified as the cytosolic binding protein ligandin,[1] which might be of significance with respect to the overall inhibition effects reported here.

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Reactivation of yeast glucose-6-phosphate dehydrogenase denaturated by saturated fatty acids[1]

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Summary. Activity of yeast glucose-6-phosphate dehydrogenase, inactivated by treatment with saturated fatty acids, can be partially restored by incubation in a medium of suitable ionic composition. The effectiveness of ions in the reactivation process is inversely related to their 'chaotropic' properties. Time-dependence of reactivation extent suggests a 2-step mechanism of enzyme inactivation and the existence of an intermediate form that aggregates through a 2nd-order reaction, producing irreversibly inactive enzyme.

Incubation of purified yeast glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49) with saturated or unsaturated fatty acids causes a time-dependent inactivation of the enzyme. The effect seems to be dependent on the following experimental conditions: temperature, protein concentration, buffer, ionic strength, fatty acid concentration and chain length, presence of ligands such as NADP(H), glucose-6-phosphate and 2′-AMP[2]. In the present work some experiments are reported about the reactivation of glucose-6-phosphate dehydrogenase inactivated by myristic acid. According to the data obtained, a plausible model for the mechanism of the denaturation process promoted by fatty acids has been postulated.

Materials and methods. NADP, glucose-6-phosphate, triethanolamine · HCl were obtained from Boehringer, Mannheim, FRG. Myristic acid and other reagents were analytical grade products from E. Merck, Darmstadt, FRG. Purified *S. cerevisiae* glucose-6-phosphate dehydrogenase (grade I) was purchased from Boehringer. Before use, the enzyme was dialyzed against 100 mM triethanolamine, pH 7.6. The enzyme was NADP-free, as shown by spectrophotometric measurements of E_{278}/E_{259} ratio and E_{340} before and after addition of glucose-6-phosphate. The concentration of the apoenzyme was estimated from the absorbance at 278 nm, assuming an $\varepsilon^{1‰}_{1cm} = 0.965$[4]. The enzyme activity was measured as described by Bücher et al.[5] at 30 °C and 340 nm, with a Gilford 3400 spectrophotometer. Incubation with potassium myristate was carried out in 50 mM triethanolamine, pH 7.6 at 30 °C. The concentration of enzyme protein was 0.1 mg/ml. In the reactivation experiments, fatty acid-treated enzyme was diluted 1000-fold in a mixture containing 0.3 mg/ml of bovine serum albumin, the indicated salt and buffer.

Results. If glucose-6-phosphate dehydrogenase is inactivated by potassium myristate or by other long-chain saturated fatty acids, reactivation can occur by simple dilution in a suitable ionic environment. Figure 1 shows the recovery of activity of the enzyme pre-treated with myristate, when diluted 1000-fold in media of increasing ionic strength and

allowed to stand 6 h. It is evident that some ions produce a reactivating action increasing with ionic strength (HPO_4^{2-}, SO_4^{2-}, Cl^-, NH_4^+, Na^+, K^+), whereas other ions produce a smaller if any action (ClO_4^-, I^-, Mg^{2+}, Ca^{2+}). The ions of the 1st kind are endowed with salting-out properties and stabilize polar structure in water, while the ions of the 2nd kind are 'chaotropic' and tend to stabilize apolar structures in water[6,7]. These data suggest the existence of a reactivable form of the enzyme, with exposed apolar residues, resulting from the myristate-induced inactivation of the native form. Time dependence of the reactivation process was evaluated at different concentrations of sodium phosphate, i.e. in an ionic environment particularly suitable for enzyme renaturation. The results are reported in figure 2. According to the reported findings, the conditions of maximal reactivation are as follows; 1000-fold dilution of the fatty acid-inactivated enzyme in a solution containing 0.3 mg/ml of bovine serum albumin, 0.4 M sodium phosphate, pH 7.5, 6 h at 30 °C.

The extent of the recovery of the enzyme activity depends also on the time of pre-treatment with fatty acid. Such a dependence can be quantified during the denaturation process, by measuring the enzyme activity both directly and after dilution in the above-reported optimal conditions. The difference between the 2 assays at each time was assumed to give an estimate of the reversibly inactivated form of the enzyme. Time courses of native and reversibly inactivated forms of the enzyme are shown in figure 3. The curve of the reactivable form describes the kinetics of the intermediate term between 2 consecutive reactions. Kinetic patterns of the same kind were obtained with other saturated fatty acids, whereas in the presence of the unsaturated oleic acid the enzyme inactivation was of the 1st order as well, but irreversible in any tested condition.

Discussion. The result of the experiment reported in figure 3 supports the hypothesis that the interaction enzyme-fatty acids gives rise to a 2-step mechanism of protein denaturation. The 1st step can be reversed by means of dilution and incubation with phosphate or other ions, as above reported; on the contrary, the 2nd step appears to be irreversible under any of the conditions tested. 2 alternative kinetic models can be suggested, uni-uni and uni-bimolecular respectively:

$$N \underset{}{\overset{k_1}{\rightleftharpoons}} R \xrightarrow{k_2} D \qquad (a)$$

$$2N \underset{}{\overset{k_1}{\rightleftharpoons}} 2R \xrightarrow{k_2'} D \qquad (b)$$

where N represents the native form, R the reversibly denaturated intermediate and D the irreversibly denaturated final product(s), resulting in (b) from the aggregation of R.

In both cases the time dependence of [N] is

$$[N] = [N_0]\, e^{-k_1 t} \qquad (c)$$

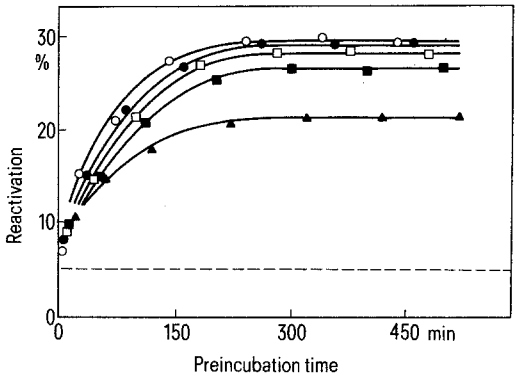

Figure 2. Time-course of the reactivation of yeast glucose-6-phosphate dehydrogenase inactivated by myristate and diluted with increasing concentrations of sodium phosphate. The enzyme, treated with myristate as indicated in figure 1, was diluted 1:1000 in a medium containing 0.3 mg/ml of bovine serum albumin and the following concentrations of sodium phosphate, pH 7.5: (▲) 50 mM; (■) 100 mM; (□) 200 mM; (●) 300 mM; (○) 400 mM. Other indications as in figure 1.

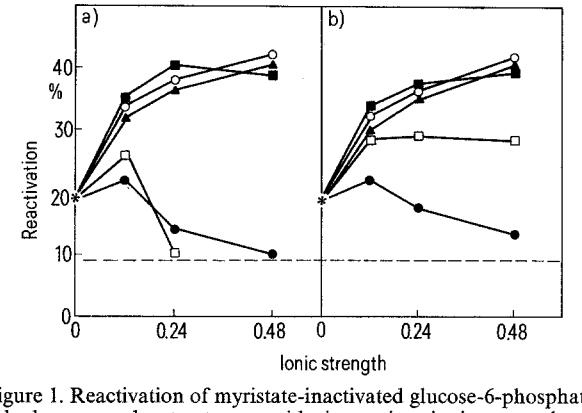

Figure 1. Reactivation of myristate-inactivated glucose-6-phosphate dehydrogenase by treatment with increasing ionic strength of different ions. The enzyme (0.1 mg/ml) was preincubated with 0.3 mM potassium myristate in 50 mM triethanolamine, pH 7.6 and 30 °C. After 15 min, samples were withdrawn, diluted 1:1000 in media containing the indicated salt + 0.3 mg/ml of bovine serum albumin in 10 mM triethanolamine, pH 7.6 and allowed to stand at 30 °C for 6 h. Reactivation is expressed as percent of the activity before treatment with myristate. Dashed line indicates the residual activity after 15 min preincubation. The native enzyme was stable in all tested conditions, except for perchlorate and iodide. In such cases the reactivation value was corrected for the loss of the activity of the corresponding control samples. *a* Sodium salts of the following anions: (○) phosphate; (▲) chloride; (■) sulphate; (●) iodide; (□) perchlorate; (*) no salt added. *b* Chloride salts of the following cations: (○) ammonium; (▲) sodium; (■) potassium; (□) magnesium; (●) calcium; (*) no salt added.

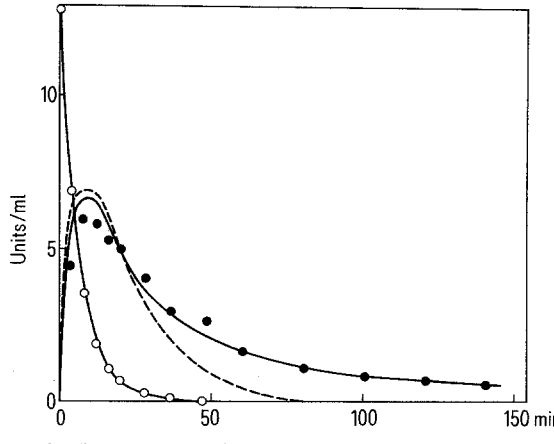

Figure 3. Time course of the native (N) and reactivable (R) forms of yeast glucose-6-phosphate dehydrogenase treated with myristate. The enzyme was treated as indicated in figure 1. At the indicated times, 2 samples were withdrawn, one for the immediate assay, the other for the reactivation procedure before assay (see text). The [R] values were calculated by difference. The enzyme concentration is expressed as $\mu moles \cdot min^{-1} \cdot ml^{-1}$ ($units \cdot ml^{-1}$). The solid lines interpolating the experimental values of N (○) and R (●) are the best fitting curves calculated according to an irreversible uni-bimolecular mechanism with $k_1 = 0.155\ min^{-1}$; $k_2' = 0.0115\ ml \cdot unit^{-1} \cdot min^{-1}$. For comparison the best fitting curve for R assuming an irreversible uni-unimolecular mechanism is also indicated (dashed line).

where $[N_0]$ is the initial concentration of N. The time dependence of [R] is different in the 2 proposed mechanisms. In the case (a) it is:

$$[R]=\frac{k_1[N_0]}{k_2-k_1}(e^{-k_1t}-e^{-k_2t}) \quad (d)$$

In the case (b) it can be obtained by integrating the following differential equation:

$$\frac{d[R]}{dt}=k_1[N_0]e^{-k_1t}-k_2'[R]^2 \quad (e)$$

which was solved by Chien[8], as follows:

$$[R]=[N_0]\sqrt{\frac{\tau}{\kappa}}\frac{iJ_1(2i\sqrt{\kappa\tau})-\beta H_1^{(1)}(2i\sqrt{\kappa\tau})}{J_0(2i\sqrt{\kappa\tau})+\beta iH_0^{(1)}(2i\sqrt{\kappa\tau})}, \quad (f)$$

where, $\tau=e^{-k_1t}$; $\kappa=[N_0]k_2'/k_1$; $\beta=\{iJ_1(2i\sqrt{\kappa})\}/\{H_1^{(1)}(2i\sqrt{\kappa})\}$; $J_0(ix)$, $-iJ_1(ix)$, $-H_1^{(1)}(ix)$ and $iH_0^{(1)}(ix)$ are Bessel functions whose values are tabulated[9].

On the basis of the time-course for the R-form reported in figure 3, the model (b) seems to be the operative one. Actually in the last part of the curve, when the N-form is practically lacking, a linear relationship between 1/[R] and time exists, that is characteristic of 2nd order kinetics. Moreover experimental data fit fairly well with equation (f), derived from the uni-bimolecular model (figure 3, solid line), while the best curve calculated according to the uni-unimolecular model (d) does not fit to the experimental data (figure 3, dashed line). Therefore, the denaturation of yeast glucose-6-phosphate dehydrogenase by saturated fatty acids apparently involves a 2-step mechanism and consequently the existence of an intermediate enzyme form (R), inactive but reactivable in a suitable medium. R molecules aggregates with each other leading to an irreversibly denaturated form of the enzyme. In the present paper the existence of such aggregates is indirectly supported by the 2nd order kinetics of the disappearance of the R-form. Actually the best fit to the experimental data was obtained with a uni-bimolecular model. On the other hand the existence of high molecular weight products was previously reported following the inactivation of the enzyme by fatty acids[2]. These aggregates presumably correspond to the products of interaction of the R-form.

For another multimeric enzyme also, i.e. swine heart fumarase, the role of the chemical composition of the reactivation medium as far as the interconversion of enzymatic forms is concerned has been pointed out[10]. Moreover, preliminary data indicate that for yeast glucose-6-phosphate dehydrogenase, as well as for fumarase, the denatured form(s) are more susceptible to specific proteolytic attack[11].

Work is in progress in our laboratory on this topic, in order to elucidate the mechanistic aspects of selective enzyme denaturation, as well as its possible physiological significance.

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Measurement of polyethylene glycol 4000: Effect of storage and freeze thawing in biological fluids

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Summary. The effects of storage and freeze-thawing on polyethylene glycol 4000 (PEG 4000) and ^{14}C PEG 4000 in a solution of NaCl (150 mmoles/l) containing varying amounts of human albumin were studied. Results showed that the analysis of both PEG 4000 and ^{14}C PEG 4000 is likely to be inaccurate in these fluids if the specimens have been freeze-stored, thawed and refrozen several times during a period of several weeks. This seems to be due to the freeze-thawing process itself rather than the actual storage. The amount of protein in the samples may increase the fall in estimated levels of polyethylene glycol observed.

During the past 3 decades polyethylene glycol of mol.wt 4000 (PEG 4000) has been used extensively in medical research as a nonabsorbable marker for estimating recovery and calculating fluid volumes in intestinal perfusion studies[1-3]. PEG 4000 remains chemically unchanged in the intestinal lumen and is nontoxic[4]. In intestinal perfusion studies PEG 4000 has been used both in a radiolabeled form (labeled with ^{14}C or ^{3}H)[5,6] and an unlabeled form. Unlabeled PEG 4000 has been measured by the turbidimetric method of Hydén[7]. Doubts have been raised, however, as to whether the chemical analysis of PEG 4000 is reliable when performed on thawed samples which have been stored frozen (−20 °C) for a variable period of time[8,9]. It is known that freeze-thawing may cause precipitation of proteins[10] and that PEG may bind to proteins in biological fluids and cause precipitation of proteins itself[11,12]. Freeze-thawing of biological samples might enhance this process and cause loss of PEG from the fluid.

We have studied the effect of storage and freeze-thawing of solutions of PEG 4000 (5 g/l) doped with ^{14}C PEG 4000 (5 μCi/l) in NaCl (150 mmoles/l) containing varying amounts of human albumin (0.0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.5, 25.0, 50.0 and 100.0 g/l, respectively). All samples were measured in duplicate and data expressed as average