pherolquinone inhibits vitamin K_1 hydroquinone-dependent protein carboxylation and that this inhibition might be involved in the mechanism of bleeding by hypervitaminosis of α-tocopherol [22].

Generally speaking, NADPH is not necessary for the inhibition of phylloquinone epoxide reductase by BHT quinone methide, salicylic acid or α-tocopherolquinone. Therefore, cytochrome P-450 and/or NADPH-cytochrome P-450 reductase do not participate in those inhibitions.

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Hepatic sulphate conjugation of triiodothyronine (T_3)

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Thyroxine (3,3′,5,5′tetraiodothyronine, T_4) is the main iodothyronine secreted by the thyroid gland. It then undergoes outer ring deiodination in peripheral tissues to produce the active hormone, 3,3′5 triiodothyronine (T_3). Besides deiodination, these iodothyronines undergo conjugation with glucuronic acid or with sulphate [1]. Though these processes have been viewed as independent of each other, sulphation of T_3 as well as T_2 appears to predispose these compounds to deiodination [2]. The sulphate esters of T_3, T_2 (3,3′-diiodothyronine) and T_1 (3′iodothyronine) could be formed *in vitro* from PAP^{35}S by sulphotransferase extracted from rat liver or monkey hepatocarcinoma cells [3]. In this paper, we present evidence that a partially purified extract of rat liver homogenate is capable of sulphate conjugation of T_3 from sodium ^{35}S-sulphate and ATP. The optimum conditions for the formation of T_3 sulphate and kinetics of this sulphate conjugation reaction were examined.

Materials and methods

Chemicals. Sodium ^{35}S-sulphate (specific radioactivity of 621.9 mCi/mmol) was purchased from New England Nuclear Corporation. The other biochemicals were from the usual commercial sources.

Enzyme preparation and assay conditions. Male Wistar rats between 150 and 200 g were used. A 20% (w/v) homogenate was prepared from freshly excised liver in 0.15 M KCl containing 3 mM dithiothreitol (DTT) using a Polytron homogenizer. The supernatant obtained after centrifugation at 108,000 *g* was passed through a PD-10 prepacked column containing Sephadex G-25M (Pharmacia). Five 1-ml fractions were collected after discarding the void volume, as recommended by the supplier. The third fraction was used for all assays.

The reaction mixture for the overall sulphate conjugation contained the following in a final volume of 100 μl: 40.2 μM $Na_2{}^{35}SO_4$ (2.5 μCi); 7 mM ATP, 7 mM Mg^{2+}, 25 μM T_3 and 0.1 mM 6 propyl-2-thiouracil (PTU; an inhibitor of deiodinase activity). The last compound was routinely added to the incubating mixture as a precautionary measure, although it is unlikely that the high-speed supernatant would contain any deiodinase activity. The reaction was started by the addition of 10 μl of the enzyme extract. At the end of 15 min of incubation, 5 or 10 μl of the incubate was spotted on a 60 cm × 0.8 cm strip of Whatman No. 1 paper. Each chromatogram was marked in 2-cm segments in pencil, with the origin 10 cm from one end. The chromatograms were developed by descending chromatography for 14–16 hr at 29–30° in 1-propanol–ammonia–water (6:3:1, by vol) [3]. Initially, all sections of the chromatograms were counted by liquid-scintillation as described for other sulphate esters [4, 5]. Two distinct radioactive peaks of R_f of 0.47 and 0.91 were observed; the former corresponded to unreacted sodium ^{35}S-sulphate and the latter was identified as $T_3{}^{35}$S-sulphate as it was not present in control experiments where boiled enzyme extract was used. Subsequently, only the radioactivity present in $T_3{}^{35}$S-sulphate was measured. Standards of $Na_2{}^{35}SO_4$ were similarly counted and the amount of $T_3{}^{35}$S-sulphate formed was extrapolated from these standards.

Protein determination. This was carried out by the method of Lowry *et al* [6].

Results

Preliminary experiments with the crude enzyme preparation showed the formation of $T_3{}^{35}$S-sulphate as a peak with an R_f of 0.91. However, control experiments in which T_3 was omitted from the assay also had some radioactivity in the corresponding position, which may approach 10–25% of the experimental value. This phenomenon has also been observed in an *in vivo* system [7]. This could be due to the presence (in the enzyme extract) of an endogenous acceptor of phenolsulphotransferase (PST). In view of this, a partial purification of the crude enzyme extract was carried out by gel-filtration using prepacked Sephadex G-25M mini-columns. Figure 1 shows that the overall sulphate conjugating profiles of three of the five eluted fractions, as well as that of the high-speed supernatant of the crude liver homogenate. Fractions 1 and 5 showed no activity. Background counts, though considerably reduced to <10% of experimental values were not completely eliminated, and therefore were subtracted from all experimental values presented in this paper. Fraction 3, which possessed the highest sulphate-conjugating activity (with specific activity averaging 183 pmoles/min/mg protein based on three assays) and the lowest background was routinely used without further purification.

Kinetic studies. The pH optimum for the three-step overall sulphate conjugation of T_3 was 8.0 using 0.05 M phosphate buffer, and the reaction proceeded linearly for 30 min of incubation. The apparent K_m values for ATP and Na_2SO_4 were, respectively, 4.5 mM and 0.1 mM and that for T_3 was 6.6 μM (Figs 2a, b and c). All these values were obtained by computer analyses using the Lineweaver–Burk plots [8]. Substrate inhibition was observed at ATP concentration above 9.67 mM.

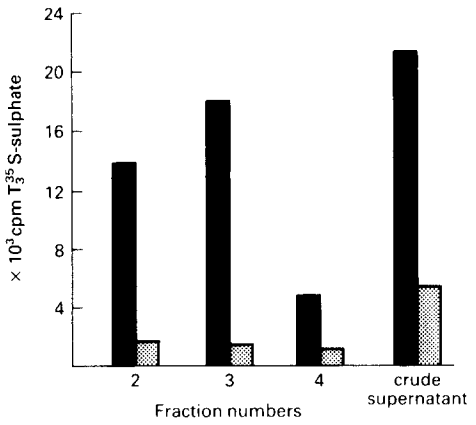

Fig. 1. Activity profile of the overall sulphate conjugation of T_3. Three of the five fractions eluted from Sephadex G-25 column (as described in text) and the high-speed supernatant of rat liver homogenate were active (solid bars) while fractions 1 and 5 showed no activity. The corresponding controls (shaded bars) in which T_3 was omitted are included for comparison.

Comparative study of the overall sulphate conjugation of T_3 by other laboratory animals. The high-speed supernatant preparations of guinea-pig, mouse and gerbil livers showed varying ability to carry out sulphate conjugation of T_3. Compared to rat liver, the ratios of their activities were 0.67:0.19:0.05. Of the rodents examined, the hamster appeared to be unable to perform this overall sulphate conjugation of T_3.

Discussion

Although sulphate conjugation of T_3 has been demonstrated by the phenolsulphotransferase reaction [3] employing a commercial preparation of PAP^{35}S, this study showed the ability of the hepatic extracts to form T_3 sulphate from inorganic sulphate and ATP. Besides being more economical compared to the PST reaction employing PAP^{35}S, the three-step reactions reflect more closely the ability of the liver to carry out sequentially the three enzymic reactions *in vivo*. As the endogenous pool of PAPS is low even in a major detoxicating organ like the liver [9], the generation of PAPS could determine the rate of overall sulphate conjugation, as has been demonstrated in human platelets [10]. The K_m value of 6.6 μM for T_3 was three orders of magnitude lower than those for ATP and sodium ^{35}S-sulphate, both of which were in the millimolar range. The K_m value of 0.1 mM for sulphate obtained in this study by the overall raction is similar to those obtained with other substrates like 1-naphthol [11], harmol [12, 13] and salicylamide [14]. The plasma concentration of inorganic sulphate measured in the rat was reported to vary from 0.15 to 0.9 mM [7, 13]. As inorganic sulphate in the liver equilibrates rapidly with that in the serum [7, 15, 16], it would appear that the liver cells have an adequate supply of inorganic sulphate for the conjugation reactions, provided it is not limited by the activities of the PAPS-generating enzymes.

The study of sulphate conjugation of T_3 was undertaken to assess the importance of sulphate conjugation. The sulphation of T_3 was reported to enhance the inner ring deiodination of T_3 [17] and reverse T_3 [18]. Though the two pathways occur in different compartments of the cell, it is possible that sulphate conjugation of T_3 in the cytosol may facilitate its transport to the cell compartment where deiodination occurs, i.e. in the endoplasmic reticulum. Furthermore, the affinity of the deiodinase for T_3 sulphate

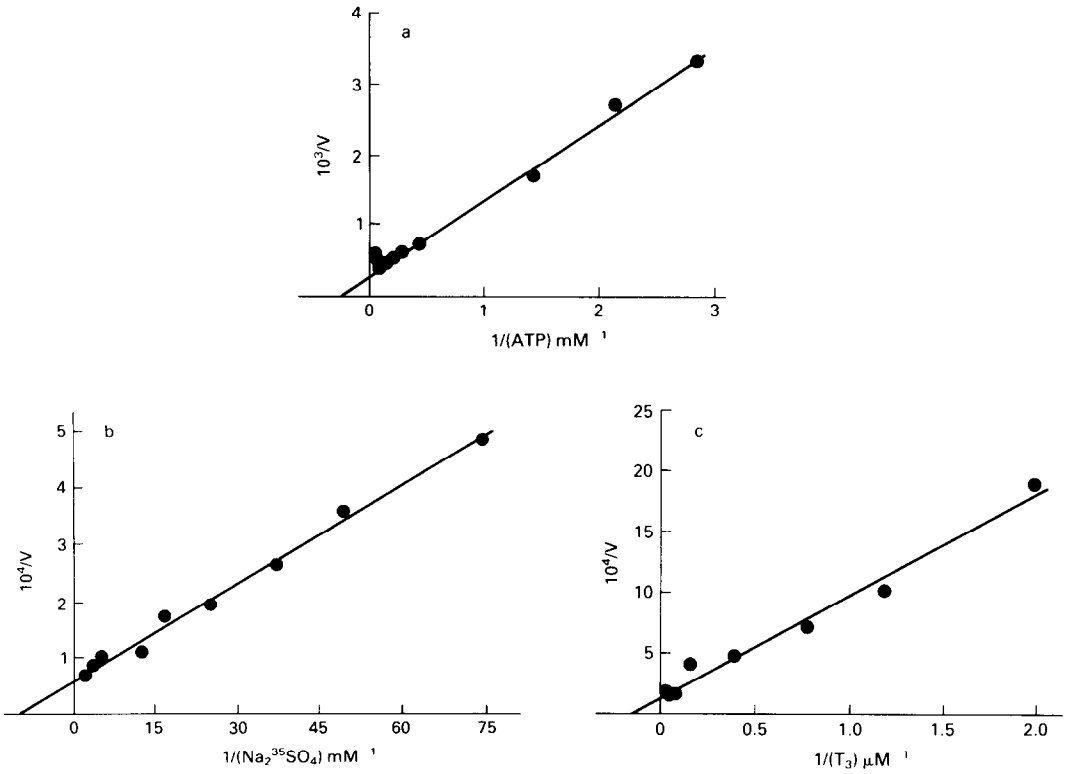

Fig. 2. Lineweaver–Burk plots of the overall sulphate conjugation of T_3. Velocity, v, was expressed in cpm/15 min in 10 μl chromatographed aliquots against (a) ATP from 0.35 to 9.33 mM, (b) sodium ^{35}S-sulphate from 0.013 to 0.4 mM, and (c) T_3 from 0.5 to 50 μM.

was reported to be higher than for T_3 [17]. Our study *in vitro* showed that T_3 could be as good a substrate of phenolsulphotransferase, having a K_m of 6.6 μM, as it is of deiodinase with a K_m of 10.7 μM [17].

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Comparative evaluation of different pathways for the liver toxicity of morphine using freshly isolated hepatocytes

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Hepatic injury has been observed in patients that are treated with narcotics. Thureson-Klein *et al.* [1] indicated that implantation of morphine pellets in mice caused hepatic fatty infiltration and increases in serum transaminases. Chang and Ho [2] and Needham *et al.* [3] showed that intraventricular injection of morphine caused an increase in serum GOT and GPT in mice and suggested that the hepatic injury by morphine was mediated via CNS mediated pathways.

Recently, Nagamatsu *et al.* [4] indicated that morphine 6-dehydrogenase in cytosolic fraction of mouse liver metabolised morphine to chemically unstable morphinone which reacted with a cellular reduced form of glutathione (GSH) and macromolecules. On the other hand, Correia *et al.* [5] showed microsomal cyt. P-450 mediated metabolic activation of morphine.

On comparing these two pathways for morphine toxicity using freshly isolated rat hepatocytes we showed the relative importance of the reaction mediated by morphine 6-dehydrogenase.

Materials and methods

Isolated hepatocytes were prepared from male rats (Wistar strain, 9–13 weeks, Nihon rat Co.) as described by Moldeus *et al.* [6] and used immediately. The viability of the hepatocytes, determined by trypan blue exclusion, was 92–97%. Hepatocytes were suspended in Krebs–Henseleit buffer supplemented with 12.5 mM HEPES (10^6 cells/ml), and these cells were incubating in rotating round-bottom siliconized glass flasks at 37° under continuous gassing with carbogen (95% O_2, 5% CO_2). Changes in viability during incubation were determined by measuring the leakage of lactate dehydrogenase from the cells [7] (initial values were 77–92%).

Cellular glutathione (GSH) was determined spectrofluorometrically [8] after centrifugation of the cell suspension at 8000 rpm for 5 sec. GSH contents were confirmed by using high performance liquid chromatography (HPLC). This method was originally described by Toyo'oka and Imai [9]. In the case of determination of morphine metabolism, the cell suspensions were frozen and thawed. After centrifugation, the supernatants were analyzed by HPLC [10].

Covalent binding of morphine to the macromolecules were determined after the incubation of hepatocytes with ^{3}H-morphine (0.5 mM, 0.15 μCi/mole) at 37°.

^{3}H-morphine (50.6 Ci/mmole) was purchased from NEN Research Products (Boston, MA). All other chemicals used were of at least reagent grade and obtained from local commercial sources.

Results and discussion

As described before [10], cellular GSH and viabilities were decreased by the addition of morphine (0.5 mM) to the hepatocyte suspension (Table 1).

Metyrapone, an inhibitor of cyt. P-450 linked drug metabolism, inhibited the GSH decrease by morphine a little (Table 1). In some cases, it slowed the rate of GSH depletion by about 30% at 30 min of incubation and inhibited the loss of cell viability by morphine. On the other hand, piperonyl butoxide, which also inhibits cyt. P-450 linked drug metabolism, slowed the GSH depletion a little, but potentiated the effects of morphine on the cell viability. Metyrapone and piperonyl butoxide themselves had no effects on the hepatocytes. SKF 525-A and α-naphthoflavone did not inhibit the toxicity at 0.1 mM. These drugs were toxic to the hepatocytes when the concentrations were higher than 0.25 mM. Morphine also decreased the GSH content of hepatocytes rapidly in which cyt. P-450 was decreased by the pretreatment of rats with $CoCl_2$ (40 mg/kg) (results were not indicated). From these results, we can interpret that the participation of the cyt. P-450 mediated pathway plays a minor role in the hepatocyte toxicity of morphine.

Table 1. Effects of various inhibitors on the decrease of cellular GSH and viability by morphine (0.5 mM)

	GSH content at 30 min (nmole/10^6 cells)	Viability at 3 hr (%)
Control	42.5 ± 1.4 (19)	62.4 ± 2.3 (18)
Morphine	8.1 ± 1.2 (19)	6.7 ± 3.3 (16)
Morphine + metyrapone	11.7 ± 2.7 (9)	13.7 ± 3.0 (8)
Morphine + naloxone	15.1 ± 4.2 (5)	43.8 ± 3.8 (5)
Morphine + naltrexone	34.1 ± 1.3 (4)	55.4 ± 7.1 (4)

These values indicate mean ± SEM and numerals in parentheses indicate the number of batch of hepatocytes.

Concentration of morphine was 0.5 mM and that of inhibitors was 1 mM.