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# QUANTITATIVE STUDIES OF SULPHATE CONJUGATION BY ISOLATED RAT LIVER CELLS USING [$^{35}$S]SULPHATE

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**Abstract**—We have developed a simple, rapid and sensitive method for the study of sulphate conjugation in isolated liver cells based on the incorporation of $^{35}$S from [$^{35}$S]sulphate. Excess [$^{35}$S]sulphate is removed by a barium precipitation procedure, leaving [$^{35}$S]sulphate conjugates in solution. We have used this method to examine the kinetics of sulphation of *N*-acetyl-*p*-aminophenol (acetaminophen), 4-nitrophenol and 1-naphthol in isolated rat liver cells. The efficiency of recovery of the sulphate conjugates was >86%. The method is applicable to the quantitative study of sulphate conjugation of any substrate which forms a sulphate conjugate that is soluble in the presence of barium, without the need for standards or radiolabelled sulphate acceptors.

Sulphate conjugation is an important metabolic pathway in the biotransformation of various xenobiotic and endogenous substances such as catecholamines, steroids and bile acids [1, 2]. The liver is the major site for sulphate conjugation and the sulphate conjugates are more water soluble than the unconjugated substrates, resulting in their rapid elimination in urine or bile. Many studies of hepatic drug metabolism *in vitro* are undertaken with subcellular fractions. These studies, however, are of limited value in predicting rates of metabolism *in vivo*; studies in isolated liver cells are more useful [3]. The study of rates of sulphate conjugation of a range of substrates in isolated liver cells requires a rapid and sensitive assay to process a large number of samples. Several methods have been described for the study of sulphate conjugation in isolated liver cells but these are specific for particular substrates [4–12]. These methods involve chromatographic separation of products with various detection systems or solvent extraction. Such methods all require standards or radiolabelled substrates for quantification, so that a new assay has to be developed for each novel substrate.

Sulphation has been studied with [$^{35}$S]sulphate in liver homogenates [13] and with 3′-phosphoadenosine-5′-phospho[$^{35}$S]sulphate (PAPS) in homogenates [14] and with purified phenolsulphotransferase [15]. Barium precipitation procedures have been used to precipitate unreacted [$^{35}$S]sulphate or [$^{35}$S]PAPS leaving $^{35}$S-labelled conjugates in solution. When [$^{35}$S]sulphate is given intravenously to rats pretreated with harmol, it is rapidly incorporated into harmol sulphate; this indicates that the PAPS pool is in rapid equilibrium with sulphate in plasma [16]. There are, however, no reports of [$^{35}$S]sulphate being used to study sulphate conjugation in isolated liver cells.

We have developed a general method, using a barium precipitation procedure, to study the kinetics of sulphation in isolated rat liver cells, and have applied this new method to the sulphation of acetaminophen, 4-nitrophenol and 1-naphthol.

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## MATERIALS AND METHODS

### *Materials*

*Animals.* Male Wistar rats (200–250 g), fed *ad lib.*, were used throughout.

*Chemicals.* $Na_2{}^{35}SO_4$ was from Amersham International (Aylesbury, U.K.). Pico-fluor 40 scintillation fluid was from Canberra Packard Ltd (Pangbourne, U.K.). Firefly lantern extract (FLE-50) and collagenase (Type IV) were from the Sigma Chemical Co. (Poole, U.K.). All other chemicals were of analytical grade or the highest grade commercially available and were obtained from Sigma or BDH Chemicals (Poole, U.K.).

### *Preparation and incubation of liver cells*

Liver cells were isolated essentially according to the two-step procedure of Seglen [17]. The Krebs–Henseleit buffer [18] used for the cell preparation and incubation consisted of 118 mM NaCl, 4.73 mM KCl, 1.18 mM $MgSO_4.7H_2O$, 1.18 mM $KH_2PO_4$ and 25 mM $NaHCO_3$; pH 7.4 when gassed with $O_2$:$CO_2$ (19:1). The liver was perfused with $Ca^{2+}$-free buffer containing 0.1 mM ethylene glycol bis-(aminoethyl)tetra-acetate (EGTA) followed by buffer containing 1.25 mM $Ca^{2+}$ and collagenase (0.5 mg/mL). The perfusion buffer also contained 20 mM glucose. The cell suspension released after rupture of the liver capsule was filtered through nylon gauze and centrifuged at 50 *g* to sediment parenchymal cells. The supernatant was removed by aspiration and the pellet washed twice with buffer. The final pellet was weighed and resuspended to give approximately 0.1–5.0 mg dry weight/mL (1 mg dry weight/mL = $2.9 \times 10^5$ cells/mL [19]) in buffer containing 1.25 mM $Ca^{2+}$ and 10 mM glucose. Dry weights were later determined by centrifugation (150 *g* for 5 min) of portions of cell suspension in preweighed 2 mL glass vials, aspiration of the

supernatants, and drying of the pellets overnight at 120° before reweighing.

Substrates, dissolved in dimethyl sulphoxide (DMSO), were added to 1 mL portions of cell suspension (final concentration of DMSO 1% v/v), together with $Na_2{}^{35}SO_4$ (0.5–5.0 $\mu$Ci/mL) in 20 mL polyethylene scintillation vials. Vials were capped and gassed with $O_2$:$CO_2$ (19:1) at room temperature. Sulphate conjugation did not occur at a measurable rate during the period at room temperature. Vials were transferred to a reciprocating water bath (Mickle Engineering, Gomshall, U.K.) at 37° and shaken at 100 cycles/min. Incubation was for 20–40 min and was terminated by centrifugation of 1 mL portions through 250 $\mu$L of silicone oil (Dow Corning 550:dinonylphthalate, 2:1 by vol.) at 12,000 *g* for 30 sec to separate cells from medium. Supernatants were heated in a boiling water bath for 5 min, before centrifugation (12,000 *g* for 1 min) to remove denatured protein, and stored at −20° for subsequent determination of sulphate conjugate by the barium method.

Cell viability was assessed by measurement of ATP contents by bioluminescence using firefly luciferin–luciferase [20] and a LKB 1251 luminometer in the integration mode. ATP contents were calculated by reference to known standards. The normal ATP content of isolated rat liver cells was 8–10 nmol/mg dry weight. The effect of added substrates on the ATP content of the cells was routinely checked.

*Barium precipiation*

The method of Foldes and Meek [15] was evaluated. One volume of deproteinized supernatant (routinely 0.1 mL) was mixed with two volumes of 0.1 M barium acetate (but see below) followed by two volumes of 0.1 M $Ba(OH)_2$ and two volumes of 0.1 M $ZnSO_4$ in a microfuge tube. The suspension was centrifuged at 12,000 *g* for 2 min and the $Ba(OH)_2$ plus $ZnSO_4$ step was repeated in the same tube without removal of the $BaSO_4$ pellet. After a second centrifugation a portion of the supernatant (routinely 0.7 mL), containing the $^{35}S$-labelled conjugate, was transferred to a scintillation vial for determination of radioactivity. The amounts of conjugated sulphate were conjugated from the specific activity of the $^{35}S$-labelled inorganic sulphate. Counts in samples from cells incubated without phenolic substrate were subtracted from those from cells with substrate. Blank determinations with $^{35}S$-labelled inorganic sulphate and buffer but without cells were also performed routinely to check the efficiency of the precipitation procedure. Omission of the barium acetate was later shown to decrease the blank; this modified method was used for kinetic determinations.

*Preparation of 4-nitrophenyl-[$^{35}S$]sulphate and 1-naphthyl-[$^{35}S$]sulphate standards*

Labelled standards were generated by incubating 1 mL portions of liver cells (10 mg dry weight/mL) with 10 $\mu$Ci of $Na_2{}^{35}SO_4$ and 25 $\mu$M 4-nitrophenol or 1-naphthol for 2 hr at 37°. The incubation medium was Krebs–Henseleit buffer containing 300 $\mu$M $MgSO_4.7H_2O$ (normally 1.18 mM) to increase the specific activity of the radiolabel. The incubations were terminated by centrifugation at 12,000 *g* for 1 min and 0.8 mL portions of supernatant were mixed with 1.6 mL of ethanol to precipitate protein. Extracts were stored at −20°. Ethanol extracts of cell incubations were centrifuged at 12,000 *g* for 1 min to remove precipitated protein before being pooled and applied to a 20 × 20 cm silica gel 60 $F_{254}$s thin layer plate with a 4 cm concentration zone. Unlabelled standards were applied to the same plate. The solvent was butanol:acetic acid:water (12:3:5 by vol.). Authentic 4-nitrophenyl-sulphate and 1-naphthyl-sulphate could be visualized with an ultraviolet lamp and ran with $R_f$ values of 0.64 and 0.60, respectively. Strips (1 cm) corresponding with the position of the standard were scraped and extracted twice with 5 mL of $H_2O$; the pooled extracts were freeze-dried. The purity of the extracts was checked by HPLC using mobile phase B (see below) for 4-nitrophenyl-sulphate and mobile phase C (see below) for 1-naphthyl-sulphate. Radioactivity was determined in the fractions collected.

*HPLC*

A System Gold high performance chromatograph (Beckman, High Wycombe, U.K.) was used throughout, comprising a model 126 binary solvent delivery module, model 167 scanning ultraviolet (UV) detector and model 506 autosampler. The outflow from the UV detector was collected with a Frac-100 fraction collector (Pharmacia). Fractions (0.5 min) were collected directly into scintillation vials (Mini 'Poly-Q' vials, Beckman) for determination of radioactivty. Analyses were performed on a 4.6 mm × 25 cm Ultra Techsphere 5 ODS column (HPLC Technology, Macclesfield, U.K.) equipped with a guard column packed with Co:Pell ODS (Whatman, Maidstone, U.K.), at a flow rate of 1 mL/min with a 100 $\mu$L injection volume.

Acetaminophen, acetaminophen glucuronide and acetaminophen sulphate were separated by a modification of the method of Adriaenssens and Prescott [9] with mobile phase A (0.1 M sodium phosphate, pH 4.5:isopropranol:98% (v/v) formic acid; 100:1.7:0.1) and quantified by peak area using the extinction at 254 nm and acetaminophen as external standard. The concentrations of acetaminophen metabolites were calculated as "acetaminophen equivalents" since their extinction coefficients are similar to each other [21]. The retention times for acetaminophen, acetaminophen glucuronide and acetaminophen sulphate were 16, 7.5 and 10.3 min, respectively. [$^{35}S$]Sulphate was not retained on the column. 4-Nitrophenyl-[$^{35}S$]sulphate was separated from [$^{35}S$]sulphate using mobile phase B (mobile phase A with 5% (v/v) methanol). The retention time of authentic 4-nitrophenyl-sulphate, using the extinction at 300 nm, was 17.5 min. 1-Naphthyl-[$^{35}S$]sulphate was separated from [$^{35}S$]sulphate using mobile phase C (mobile phase A with 30% (v/v) methanol). The retention time of authentic 1-naphthyl sulphate using the extinction at 300 nm, was 14.5 min.

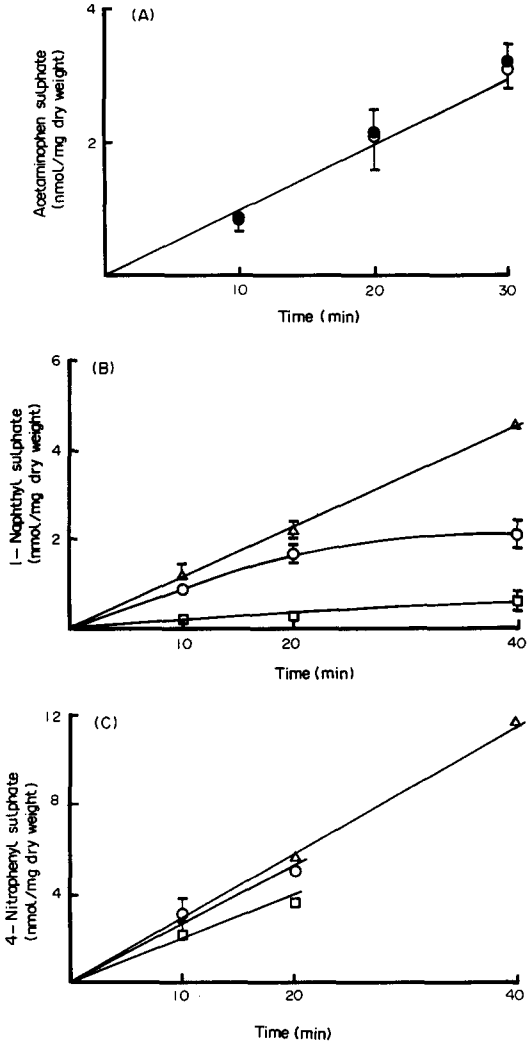


Fig. 1. The time course of sulphation by isolated rat liver cells. Sulphate conjugate released into the incubation medium was measured by the barium method. (A) Cells (1 mg dry weight/mL) were incubated with 40 $\mu$M acetaminophen (○) or were preincubated for 10 min with [$^{35}$S]sulphate before incubation with 40 $\mu$M acetaminophen (●). Values are means ± SD, N = 3 for a representative experiment. (B) Cells (0.12 mg dry weight/mL) were incubated with 0.1 (□), 0.3 (○) or 1 $\mu$M (△) 1-naphthol for 0–40 min. Values are means ± range of duplicate determinations in a representative experiment. (C) Cells (0.5 mg dry weight/mL) were incubated with 3 (□), 5 (○) or 10 $\mu$M (△) 4-nitrophenol for 0–20 or 0–40 min. Values are means ± range of duplicate determinations in a representative experiment (1 mg dry weight = $2.9 \times 10^5$ cells [19]).

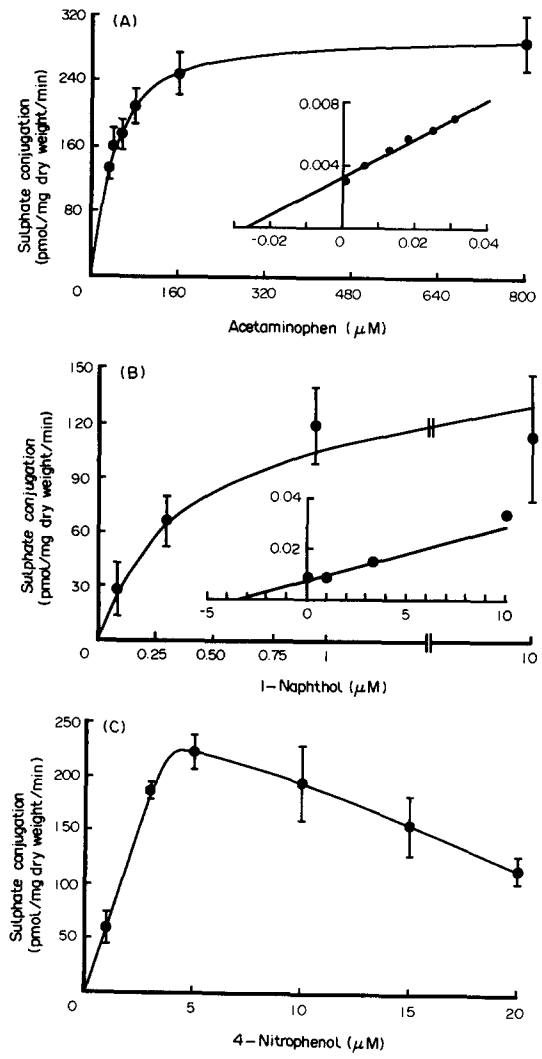


Fig. 2. The concentration dependence of sulphation by isolated rat liver cells, determined by the barium precipitation method. The conditions used were (A) 0.96–1.10 mg dry weight/mL cells and 20 min incubation, (B) 0.12–0.30 mg dry weight/mL cells and 10 or 20 min incubation and (C) 0.50–0.59 mg dry weight/mL cells and 20 min incubation. The insets show $1/v$ against $1/[S]$ and the lines correspond to the kinetic parameters obtained as described in the text. Results are means ± SEM, N = 3 (A,C) or 4 (B).

### *Kinetic studies*

The concentration dependence of sulphate conjugation with acetaminophen, 4-nitrophenol and 1-naphthol as substrates was studied using the barium method. The conditions used were such that reaction rates were linear with time and not limited by depletion of substrates. For sulphation of acetominophen and 1-naphthol, kinetic parameters were determined by computer fitting of results from individual experiments to the Michaelis–Menten equation by non-linear least squares regression. For sulphation of 4-nitrophenol the maximum rate and substrate concentration giving half this rate ($EC_{50}$) were determined graphically for individual experiments.

## RESULTS

### *Evaluation of the barium method*

The barium precipitation procedure was used with

Table 1. Kinetic parameters for the sulphation of acetaminophen,1-naphthol and 4-nitrophenol determined from the data in Fig. 1

| | $V_{max}$ or maximum rate (pmol/mg dry weight/min) | $K_m$ or $EC_{50}$ ($\mu$M) |
|---|---|---|
| Acetaminophen | 302 ± 38 | 38 ± 3 |
| 1-Naphthol | 137 ± 33 | 0.31 ± 0.05 |
| 4-Nitrophenol | 233 ± 19 | 1.8 ± 0.3 |

For sulphation of acetaminophen and 1-naphthol, kinetic parameters were determined by computer fitting of results from individual experiments to the Michaelis–Menten equation by non-linear least squares regression. For sulphation of 4-nitrophenol the maximum rate and substrate concentration giving half this rate ($EC_{50}$) were determined graphically for individual experiments. Results are means ± SEM from 3 or 4 experiments.

incubations containing 10–133 pmol of 1-naphthyl-[$^{35}$S]sulphate or 5–71 pmol of 4-nitrophenyl-[$^{35}$S]sulphate. The recoveries in the supernatants were 92 ± 4 and 93 ± 3% (mean ± SD, N = 10) of the conjugates, respectively. The recovery with 10–133 pmol of 1-naphthyl-[$^{35}$S]sulphate was not changed by addition of 1 $\mu$mol unlabelled 1-naphthyl-sulphate to the samples before precipitation (91 ± 3%, mean ± SD, N = 10). The recovery with 52 pmol 4-nitrophenyl-[$^{35}$S]sulphate (92 ± 0%) was not changed by the addition of 1 $\mu$mol unlabelled 4-nitrophenyl-sulphate (92 ± 3%) or 10 $\mu$mol $Na_2SO_4$ (92 ± 2%) to the samples before precipitation (means ± range of duplicate determinations from one experiment). In a control experiment with [$^{35}$S]sulphate (10 $\mu$Ci/mL) 99.98 ± 0.01% (SD, N = 6) of the radiolabel was precipitated.

The recovery of acetaminophen sulphate following the barium procedure was checked by simultaneous comparison of the barium method, separation by HPLC with radiochemical determination of $^{35}$S and by HPLC with UV detection. Supernatants from incubations of liver cells with acetaminophen (800 $\mu$M; incubation time 20 min) contained 6.6 ± 0.2, 6.5 ± 0.1 and 6.6 ± 0.1 $\mu$M acetaminophen sulphate (means ± SD from triplicate incubations in a representative experiment) by the three methods respectively. These results confirm that the specific activity of the sulphate conjugate is the same as that of the [$^{35}$S]sulphate. In the kinetic studies using the barium procedure all recoveries were taken as 100%.

*Kinetic studies of sulphate conjugation*

Preliminary studies of the kinetics of sulphation of 4-nitrophenol and 1-naphthol, showed that, when the initial substrate concentration was in the micromolar range, metabolism was rapid. With 1.18 mM sulphate in the standard Krebs–Henseleit buffer, sufficient incorporation of radiolabel was only obtained when a high specific activity of [$^{35}$S]sulphate was employed. Because the sensitivity of the method depends on the efficiency of the precipitation procedure, experiments were performed to try and decrease further the [$^{35}$S]sulphate left in the supernatant. Repetition of the barium plus zinc precipitation a third time had no effect. Omission of the barium acetate addition, however, decreased blank values. With 2.5 $\mu$Ci of [$^{35}$S]sulphate in 1 mL incubations, the zero time blank fell from approximately 400 dpm to approximately 100 dpm per 0.1 mL sample, i.e. 0.04% of the total counts. Samples assayed with and without the acetate step gave similar results after blanks were subtracted. The modified method (without barium acetate) has a sensitivity of approximately 0.2 $\mu$M organic sulphate in the incubation; this is adequate for kinetic studies.

Incubation of isolated rat liver cells with acetaminophen, 4-nitrophenol and 1-naphthol resulted in the time-dependent formation of the corresponding sulphate conjugate, measured in the incubation medium. By using a suitable amount of cells and incubation time, linear rates of sulphate conjugate formation could be achieved (Fig. 1). Preincubation of the cells with [$^{35}$S]sulphate for 10 min before adding acetaminophen did not change the rate of sulphate conjugate formation (Fig. 1A). The reduced rate seen with 0.3 $\mu$M 1-naphthol (Fig. 1B) between 20 and 40 min is probably due to substrate depletion. The concentration-dependence of sulphate conjugate formation from the three substrates is shown in Fig. 2. With acetaminophen and 1-naphthol, the behaviour appeared to be as predicted by Michaelis–Menten kinetics over the concentration range shown (Fig. 2A and B). In contrast, sulphation of 4-nitrophenol exhibited apparent substrate inhibition above a concentration of 5 $\mu$M (Fig. 2C). Sulphation of 1-naphthol also showed apparent substrate inhibition above 10 $\mu$M (data not shown). The kinetic parameters are summarized in Table 1.

## DISCUSSION

There is a need for simple and robust assays for rates of drug metabolism in cell preparations *in vitro*. Such assays should not be dependent on the availability of radiolabelled drugs because these are either not readily available or prepared or are of low specific activity. The method described in this paper offers (i) a potentially high sensitivity because of the relatively high specific acitivities possible with sulphate, (ii) a practically simple procedure which can be applied to large numbers of independent

samples simultaneously, and (iii) a method which can in principle be applied to human liver preparations. This method is applicable to the sulphation of any compound which forms a sulphate conjugate which is soluble in the presence of barium ions and hence offers an advantage over other methods which are specific for a particular substrate, technically more involved or time-consuming.

In the present study, the sulphates of acetaminophen, 4-nitrophenol and 1-naphthol were all recovered with efficiencies better than 86%; however, studies using similar barium precipitation techniques have reported lower percentage recoveries with sulphate conjugates of steroids (66–96%; [13]) or with catecholamine metabolites (25–42%;[15]). The measurement of *absolute* rates of sulphation of novel substrates therefore requires that recoveries are determined for each substance. This is not a very complex procedure as described here; *relative* rates, which are suitable for many purposes, are, of course, not dependent on this measurement.

The liver is the major site of both sulphation and glucuronidation and a number of substrates are both sulphated and glucuronidated. Some studies show that sulphation is more important than glucuronidation only at low substrate concentrations, both *in vivo* [22] and *in vitro* [6, 12, 22–25] but thorough kinetic studies need to be undertaken to confirm if this is the case for all substrates. Many studies have been performed with subcellular fractions but as the rate of metabolism is affected by a number of factors such as transport of substrates to the active site of the enzymes involved, the availability of cofactors and the presence of competing endogenous substrates studies in isolated liver cells should be of more value in predicting routes and rates of metabolism *in vivo*. The assay of sulphate conjugation by liver cells described here should be of use in such studies.

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