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KINETICS OF SULPHATE CONJUGATION IN EXTRACTS OF HUMAN FOETAL LIVER CELLS IN CULTURE

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Abstract—Sulphate conjugation was investigated using extracts of human foetal liver cells in culture. The three reactions which are involved in sulphate conjugation were measured singly or in combination: they are (i) the PAPS generation catalyzed by ATP-sulphurylase and adenosine 5′-phosphosulphate (APS)-kinase, (ii) the phenolsulphotransferase (PST) reaction, and (iii) the overall sulphate conjugation which comprises the above three reactions. All were radiometric assays employing PAP^{35}S or sodium ^{35}sulphate. N-acetyldopamine (NADA) was the substrate of choice although the reactions were also demonstrated with dopamine and 1-naphthol. Kinetic studies with NADA showed two pH optima of 6.7 and 8.6 for the overall sulphate conjugation and the PST reaction while the PAPS generation occurred maximally at pH 8.0. The apparent K_m value for NADA measured by both the PST and the overall sulphate conjugation reactions was the same, being 38 μM, while that for inorganic sulphate, of 107 μM and 240 μM (measured by the overall sulphate conjugation reactions and by PAPS generation, respectively) was two orders of magnitude higher than that of PAPS, which was 2.57 μM. It was possible to maintain a relatively constant level of the three activities of sulphate conjugation in confluent, quiescent cultures. The importance of sulphate conjugation for detoxification in foetal cells is discussed.

Phenolsulphotransferase (PST, EC. 2.8.2.1)‡ is the enzyme responsible for the transfer of "active sulphate" to many endogenous as well as exogenous substrates. Previous studies of detoxication reactions catalyzed by PST, UDP-glucuronyltransferase and glutathione-*S*-transferase have been reported with primary monolayer cultures of adult hepatocytes from various animals and from humans [1, 2]. This study was confined to the three reactions which participate in sulphate conjugation: namely (i) the PAPS generation involving the combined actions of ATP sulphurylase and APS-kinase, (ii) the PST reaction, and (iii) the overall sulphate conjugation consisting of all the three enzymic reactions mentioned above. The assay conditions were essentially similar to those reported previously [3, 4]. A systematic study of the kinetics of these reactions was carried out and their activity profiles analyzed for cells in different stages of culture. N-acetyldopamine (NADA) was the preferred substrate as it has been found to be a good acceptor of PST in several systems [3–6]. It is a biogenic amine reported to be present in normal kidney and human urine [7]. However, both dopamine and naphthol were also found to be sulphate conjugated by these cells in culture. The use of human cells in culture as model systems for studies of sulphate conjugation will be discussed.

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‡ Abbreviations used: PST, phenolsulphotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; APS-kinase, adenosine 5′-phosphosulphate-kinase; NADA, N-acetyldopamine.

MATERIALS AND METHOD

Materials. Sodium ^{35}sulphate (three batches of specific radioactivities of 717.39, 574.58 and 720.02 mCi/mmol) and 3′-phosphoadenosine 5′-phospho^{35}sulphate (PAP^{35}S) of specific radioactivity of 0.90 Ci/mmol were purchased from New England Nuclear Corporation. The other chemicals were of analytical grade from the usual commercial sources.

Liver tissue acquisition and conditions of culture. Livers from second trimester prostaglandin-induced abortuses were removed asceptically and cut into small 2–3 mm cubes in cold 0.01 M citric acid in physiological saline, adjusted to pH 7.6 with Tris at 37° (cist solution). The liver cubes were washed with cist solution until the supernatant was clear before being stirred in the "Melnick" (trypsinizing flask with reservoir and side arm above reservoir) with prewarmed 0.1 M collagenase (Sigma, Type I) in cist solution. Digestion was maintained at 37° and the cells harvested on replacement with fresh collagenase–cist solution at about half-hour intervals. A 50 ml tube was used to collect each harvest topped up with cold cist solution. The tube was allowed to stand undisturbed for the larger particles to settle to the bottom before having the supernatant aspirated. The cells were washed several times in this way with cist solution until the supernatant was clear before being pooled. The total cell population in the pooled harvests was estimated by a standard linear regression line of turbidity reading in an EEL colorimeter and actual cell counts in a Neubauer chamber [8]. Five to 10 million cells were plated per 25 cm^2 culture flask (Lux) in Dulbecco's modified Eagle

Medium (Flow Lab.) supplemented with 10% foetal bovine serum (Flow Lab.). Confluent cultures were trypsinized with 0.025% trypsin in cist solution (Gibco) and resuspended in growth medium, which was replaced with 10% dimethyl sulphoxide (Sigma) in foetal bovine serum (Flow Lab.) before a slow freezing down in 2 ml Wheaton ampoules placed inside a taped expanded polystyrene box at −30°. On the next day, frozen ampoules were stored at −196° in liquid nitrogen. When required, the ampoules were retrieved from liquid nitrogen and quickly placed in a water bath at 37° with vigorous agitation for a rapid thaw. Cells were washed with growth medium and replated. The growth medium was changed each day until the cells were used.

Preparation of extract from human foetal cells for enzyme assays. The cells were resuspended in the culture medium and centrifuged at 15,000 *g* for 15 min. The pellet was washed with 0.15 M KCl containing 3 mM dithiothreitol (DTT) and recentrifuged as before. The wet weight of the cells was noted and a 5% (w/v) suspension in 0.15 M KCl–3 mM DTT was prepared. The suspension was stored in small aliquots of 0.2 ml and kept frozen at −80°. Thawing of the aliquots resulted in the release of the cell contents and as all the three enzymes involved in sulphate conjugation are soluble proteins, the cell extracts so obtained were used directly for their assays.

Preparation of rat liver extract for the assay of PAPS generation. Male Wistar rats between 150 to 200 g were used. A 20% homogenate was prepared from freshly excised liver in 0.15 M KCl–3 mM DTT using a Polytron homogenizer. The supernatant obtained after centrifugation at 108,000 *g* was used.

Enzyme assays. Essentially, the procedures reported earlier [3, 4] were followed with little modification.

(a) *PAPS generation:* The incubate contained the following: 15 μl extract of cultured foetal cells containing 25–35 μg protein, measured by the Lowry procedure [9], 8 mM ATP, 7 mM Mg^{2+}, 0.44 mM or 0.69 mM sodium ^{35}sulphate (for the batches with specific radioactivities of 574.58 and 720.02 mCi/mmol, respectively) and 50 mM KH_2PO_4–NaOH buffer, pH 8 in a total volume of 50 μl. PAPS generation was allowed to proceed for 15 min and the reaction was terminated by boiling for 1 min in a water bath. $PAP^{35}S$ so formed was measured by the transfer of ^{35}S to NADA by PST present in the high-speed supernatant preparation of rat liver. The latter was added as a mixture containing rat liver extract, 0.1 M EDTA (adjusted to pH 8.0) and 0.1 M pyrophosphate in a ratio of 1:2:3 by volume. The assay incubate for the PST reaction thus contained 50 μl of the boiled incubate, 77 μM NADA and 10 μl of the mixture of rat liver extract, EDTA and pyrophosphate in a total final volume of 65 μl. The details and rationale for the inclusion of EDTA and pyrophosphate in the assay of PAPS generation have been published [4].

(b) *PST assay:* This reaction was studied using NADA as the substrate. The reaction mixture contained the following made up to a final volume of 50 μl with 50 mM KH_2PO_4–NaOH buffer, pH 6.5: 100 μM NADA, 50 μM dopamine or 100 μM 1-naphthol; 1.32 μM or 26.4 μM $PAP^{35}S$ used in the earlier and later part of this study, respectively. The short half-life of ^{35}S necessitates the use of progressively higher concentration of $PAP^{35}S$ with time. The reaction was started with 10 or 15 μl of the cell extract, containing 25–35 μg protein. After 5 min of incubation at 37°, 10 μl of the assay mixture was spotted on a strip of Whatman No. 1 paper.

(c) *The overall sulphate conjugation.* A typical reaction mixture of final volume of 50 μl contained 100 μM NADA, 0.28, 0.22 or 0.69 mM $Na_2{}^{35}SO_4$ (for the three batches employed in this study); 5 mM ATP; 5 mM Mg^{2+}, 50 mM glycine–NaOH buffer, pH 8.5. The reaction was started by the addition of 10 μl of the cell extract as above, and after 30 min of incubation, 10 μl of the reacted incubate was chromatographed.

In all the above three assays, the formation of NADA^{35}sulphate which has an r.f. of 0.7 on paper chromatograms developed in isopropanol–ammonia–water (8:1:1, by vol.) was measured by liquid scintillation counting and quantitated from standards of sodium ^{35}sulphate or $PAP^{35}S$, counted in a similar manner.

RESULTS

To release the soluble enzymes which participate in sulphate conjugation from the cultured cells, different ways of freezing and thawing were examined, namely (i) rapid freezing in liquid nitrogen followed by thawing, (ii) slow freezing and thawing once, twice and three times. Except for the last method which led to about 22% loss in PST activity and about 32% of the overall sulphate conjugation, the other procedures were equally suitable. Thus routinely, all aliquots were subjected to slow freezing and thawing once only.

Kinetics data

All apparent K_m values below were obtained by computer analysis of data using the Lineweaver–Burk plots [10].

PAPS generation

PAPS generation by human foetal liver cells in culture showed a broad peak of activity at pH 8.0 (Fig. 1) and the reaction proceeded linearly for 15 min of incubation and thereafter increased progressively for 60 min. The dependence of PAPS formation on the concentrations of ATP is shown in Fig. 2A; slight inhibition was observed above 9 mM ATP. The apparent K_m value for sodium sulphate was 239.7 μM (Fig. 3a).

The PST activity

The PST activity in human foetal liver cells was a fast reaction, even when measured at PAPS concentration below its apparent K_m value of 2.57 μM (Fig. 4) as normally employed in the standard essays. The apparent K_m for NADA was 38 μM which was identical to the value obtained by the overall sulphate conjugation reactions (Fig. 5). The reaction was linear for 5 min and continued to increase for 30 min

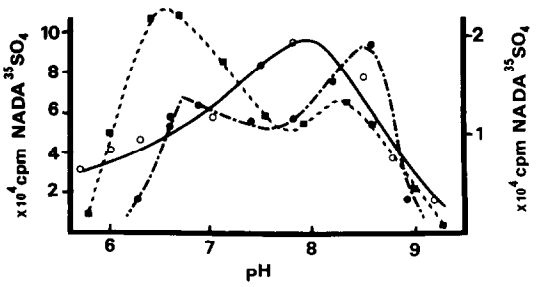

Fig. 1. pH activity profiles of (A) PAPS generation (○——○) represented by the left vertical axis, (B) PST reaction (■---■) and (C) the overall sulphate conjugation (●---●), represented by the right vertical axis. 50 mM KH_2PO_4–NaOH buffer was used for pH values < 8.4 and 50 mM glycine–NaOH buffer for pH > 8.4.

of incubation. The pH activity curve was biphasic (Fig. 1) with PST activity at pH 6.7 being higher than that at 8.6 by a factor of 1.6. Activities of PST from different batches of confluent, quiescent cells in culture were measured and compared. Generally, confluency was reached between 9 to 12 days, and the PST activity appeared relatively constant during this quiescent phase (Table 1). Parallel experiments with dopamine and 1-naphthol as substrates also demonstrated the formation of dopamine ^{35}sulphate (r.f. of 0.44) and 1-naphthol ^{35}sulphate (r.f. of 0.86) in the same solvent system used for separating NADA ^{35}sulphate.

The overall sulphate conjugation reactions

As demonstrated above, the extracts prepared from human foetal liver cells in culture were able to synthesize PAPS *in vitro* and to transfer ^{35}S from PAP^{35}S to NADA by the PST reaction. It is evident that these cells possess a full complement of the three enzymes, namely ATP sulphurylase, APS-kinase and PST for sulphate conjugation. This was demonstrated by the formation of NADA^{35}sulphate from ATP and inorganic ^{35}sulphate together with the inclusion of NADA in the incubate. The overall reaction was linear for at least 60 min of incubation, similar to the other systems examined earlier [3, 5, 6]. Like the PST reaction, two pH activity peaks were observed for the overall sulphate conjugation (Fig. 1) but the activity at pH 8.6 was higher than that at pH 6.7 by 1.5-fold. Variation of ATP concentrations did not produce classical Michaelis–Menten kinetics (Fig. 2b). As ATP is a substrate of the first two reactions, the data obtained served to determine the concentration of ATP for this assay.

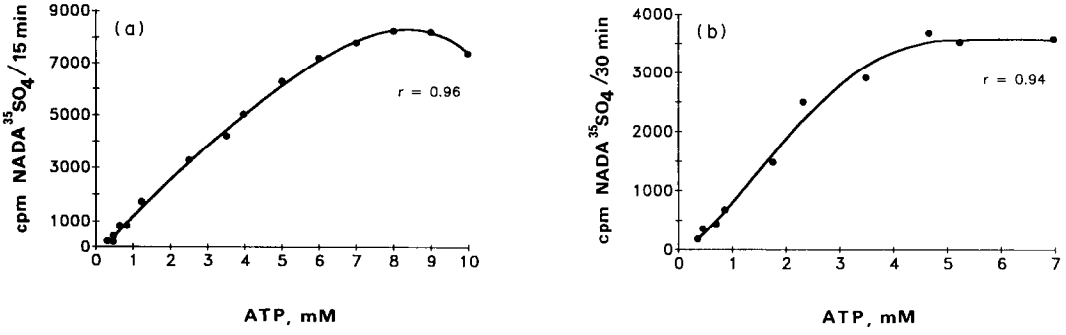

Fig. 2. Variation of ATP concentration on (a) PAPS generation and (b) the overall sulphate conjugation of N-acetyldopamine (NADA).

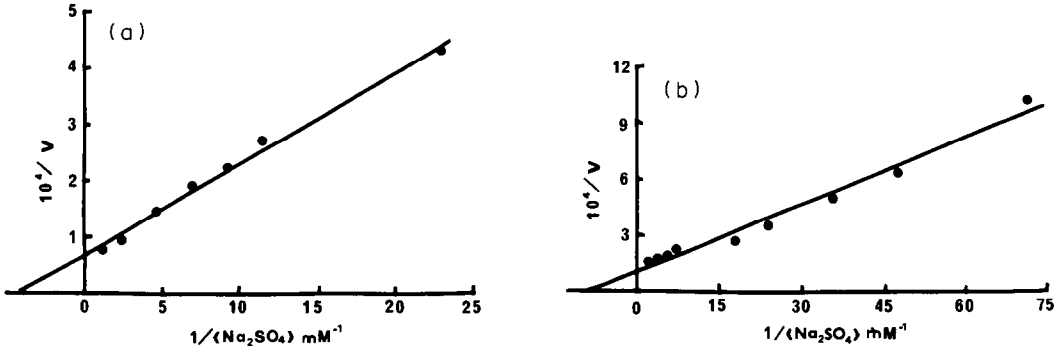

Fig. 3. Lineweaver–Burk plots of (a) PAPS generation, where velocity v is expressed in cpm NADA ^{35}sulphate formed/15 min against sodium ^{35}sulphate between 43.5 and 870 μM and (b) the overall sulphate conjugation where velocity v is expressed in cpm NADA ^{35}sulphate formed/30 min for sodium ^{35}sulphate between 13.9 and 558 μM.

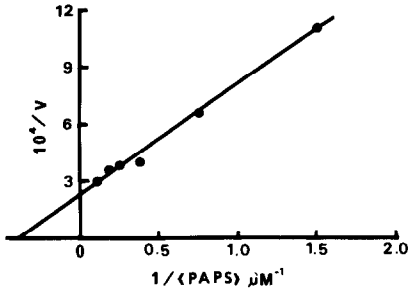

Fig. 4. Lineweaver–Burk plot of velocity v in cpm NADA ^{35}sulphate/10 min against PAP^{35}S concentration from 0.66 μM to 8.8 μM.

The apparent K_m value for sodium sulphate by the overall sulphate conjugation reactions was 107 μM (Fig. 3b).

Enzyme activities in different stages and batches of culture

The activity profiles of PST, PAPS generation and overall sulphate conjugation by these human foetal liver cells showed higher values for the initial five days in culture (Fig. 6). Lower but constant activities were observed in confluent quiescent cells (Fig. 6). This was also observed in the earlier set of data accrued for the PST activity (Table 1), the lower specific activity of which was possibly due to the lower concentration of PAP^{35}S employed (1.32 μM) compared to that (of 26.4 μM) used in the set of experiments shown in Fig. 6. Thus homogenates of human foetal liver cells in culture exhibited a relatively constant level of sulphate conjugating activity during confluency.

DISCUSSION

Cultures of hepatocytes have been used increasingly in pharmacological and toxicological studies. However, sulphotransferase activity towards 1-naphthol declined rapidly by 70% within 72 hr in cultured rat hepatocytes [1], while no activity was detected in culture of adult human hepatocytes [2]. PST activity was not/or just detectable in all the human and mammalian cell lines in culture [11] and in human lung cell lines [12]. It would therefore be difficult to carry out any systematic study of sulphate conjugation in such preparations. In this study, human foetal cells in culture were demonstrated to be capable of generating the "active sulphate" as well as transferring it to three different acceptors to form their respective sulphate esters. In essence these cells express the following enzyme activities: ATP sulphurylase, APS-kinase and phenolsulphotransferase (PST). Extracts of these cultured cells showed high potential of PAPS generation (Fig. 6). From the pH activity profiles of these reactions (Fig. 1), it would appear that PAPS-generation, which exhibits activity between pH 7.5 and 8.6, contributes to the high rate of overall sulphate conjugation at pH 8.5, in spite of the lower PST activity at this alkaline pH. Dependence of sulphate conjugation on PAPS generation has previously been shown in human platelets [4]. A comparison of the specific activities in which PAPS was added directly to the incubate (i.e. in the PST assay) with the overall sulphate conjugation in which PAPS was generated from ATP, Mg^{2+} and sodium ^{35}sulphate (Fig. 6) showed that preformed PAPS was more effective even when

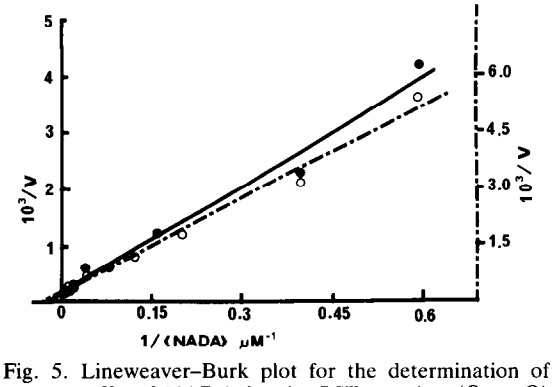

Fig. 5. Lineweaver–Burk plot for the determination of apparent K_m of NADA by the PST reaction (○---○) where velocity v is expressed in cpm NADA ^{35}sulphate/15 min between 1.67 μM to 100 μM NADA and the overall sulphate conjugation reaction (●---●) in cpm NADA ^{35}sulphate/30 min for 1.67 to 250 μM NADA.

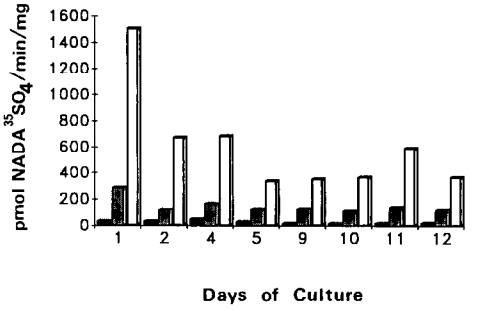

Fig. 6. The specific activities (expressed in cpm NADA$^{35}SO_4$ formed/min/mg protein) of PAPS generation (open bars), the PST reaction (shaded bars) and the overall sulphate conjugation (solid bars) measured in extracts of human liver foetal cells maintained up to 12 days of culture. Days 9 to 12 represent the confluent phase of culture. In this series, the PST activity was assayed at 26.4 μM PAP^{35}S.

Table 1. PST activity of different batches of confluent, quiescent human foetal liver cells in culture

Batch	Specific activity expressed in pmol NADA ^{35}sulphate/min/mg protein*
1	77.0
2	73.2
3	61.6
4	64.1
5	75.6
	Mean ± SD = 70.3 ± 7.0

* The PST assay was carried out with 1.32 μM PAP^{35}S and activity was determined for a 5 min assay.

it was added at a suboptimal concentration of 1.32 μM (cf. K_m of 2.57 μM). Similar observations were made earlier with human platelets [3, 4] and cultured aortic tissues and cells [13].

Although there was a decline in activities of PAPS generation, PST and overall sulphate conjugation during the quiescent phase of cell culture compared to the initial five days of culture, the ability to maintain these activities at a relatively constant level permits detailed studies of the kinetics of sulphate conjugation. The lower level of activities was compensated by a larger number of cells during this period of cell culture. Since these cells in culture are able to express complete overall sulphate conjugating activity when provided with the necessary precursors, they could be useful as test systems for evaluating the potential cytotoxicity of chemicals to the human foetal liver which would otherwise be difficult or impossible to examine. It is possible that only foetal liver cells exhibit this capability as adult human hepatocytes were unable to form sulphate conjugates [1] and activity of chondroitin sulphotransferase was high in foetal serum compared to adult serum [14]. In contrast to the sulphate conjugating activity demonstrated in foetal cells in this paper, other detoxicating enzymes like acetyltransferase, glutathione-*S*-transferase and glucuronyl transferase appear to be considerably lower in foetal compared to adult tissues [15–17]. The implication of sulphate conjugation as an important mode of phase II biotransformation process in the foetus merits further investigations.

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