Sulphation of L-tyrosine in mammalian cells: a comparative study

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Chang liver cells, Caco-2 human intestinal epithelial cells and Madin–Darby canine kidney (MDCK) cells, labelled with [³⁵S]sulphate in the presence of different concentrations of cycloheximide, produced 87.7–95.3%, 35.8–41.1% and 23.2–25.9%, respectively, of the amounts of free tyrosine O-[³⁵S]-sulphate (Tyr[³⁵S]) formed by corresponding cells labelled in the absence of cycloheximide. Homogenates prepared from the three kinds of cells showed the presence of enzymic activities catalysing the sulphation of L-tyrosine, with specific activities in

the order: Caco-2 cells > MDCK cells > Chang liver cells. In all three cases, most of the ‘tyrosine sulphotransferase’ activity was found in the cytosolic fraction, indicating the enzyme to be a cytosolic protein. A tyrosine-dependence experiment revealed that, for all three kinds of cells labelled with [³⁵S]sulphate, the production of free Tyr[³⁵S] was proportional to the concentration of L-tyrosine present in the culture medium. These results imply an involvement of sulphation in removing excess intracellular L-tyrosine.

INTRODUCTION

Enzymatic sulphation plays a variety of functional roles in mammalian physiology [1]. Upon sulphation, some peptides or proteins may undergo changes in their biological activities, thereby fulfilling different biochemical/physiological needs [1,2]. For low-molecular-mass xenobiotics or certain endogenous compounds such as steroids, catecholamines, bile acids and thyroid hormones, sulphation is believed to increase the water-solubility and facilitate their excretion from the body by endowing them with (additional) charged properties [1,3,4]. In relation to this latter respect, the functional relevance of the excretion of free tyrosine O-sulphate (TyrS) in mammalian urine has remained an intriguing question for the last 40 years.

Free TyrS was first shown to be excreted in human urine in 1955 by Tallan et al. [5]. Similar findings were subsequently reported for other mammals, including mouse, rat and rabbit [6,7]. Much effort has since been made to investigate the biochemical origin of the free TyrS excreted. Two distinct mechanisms for the generation of free TyrS have been considered: the sulphation *de novo* of the amino acid L-tyrosine, and the degradation of tyrosine-sulphated proteins, releasing constituent TyrS in the free form. With respect to the first of these two mechanisms, studies using cell homogenates or purified aryl sulphotransferases persistently failed to reveal the enzymic activity catalysing the sulphation of L-tyrosine [8–11]. The repeated failure led to the postulation that the free TyrS excreted in mammalian urine may arise from the turnover of tyrosine-sulphated proteins *in vivo* [7,12,13]. Indeed, free Tyr[³⁵S] was shown to be generated and released when tyrosine-[³⁵S]sulphated peptides or proteins were either injected into rabbits [12] or added to the medium of cultured cells [14]. These results, combined with those showing that TyrS cannot be used for protein synthesis [15] or efficiently hydrolysed by mammalian arylsulphatases [16,17], have further strengthened the viewpoint that the free TyrS excreted is derived exclusively from the degradation of tyrosine-sulphated proteins. The large amount of excreted TyrS, reported to be approx.

28 mg/day per normal adult human [5], however, continues to arouse the question concerning the need for the rapid turnover of tyrosine-sulphated proteins, if the latter truly is the sole source of the former. In view of the recent finding that sulphation of L-tyrosine takes place in the green alga *Euglena gracilis* [15], we decided to re-examine the production of free TyrS via sulphation *de novo* of L-tyrosine in mammalian cells by employing a radioactive-labelling technique.

We report here the evidence indicating the formation of free TyrS by sulphation *de novo* of L-tyrosine in three mammalian cell lines, Chang (human) liver cells, Caco-2 human intestinal epithelial cells and Madin–Darby canine kidney (MDCK) cells. Results showing (i) the production of free TyrS by cells incubated in media containing cycloheximide, a protein-synthesis inhibitor [18], (ii) the existence of the enzymic activity catalysing the sulphation of L-tyrosine, and (iii) the dependence of the production of free TyrS on the concentration of L-tyrosine in the culture medium are presented.

MATERIALS AND METHODS

Materials

Cycloheximide, phenylmethanesulphonyl fluoride, aprotinin, antipain, benzamidine, soybean trypsin inhibitor, Hepes, ninhydrin, ATP, AMP, minimum essential medium (MEM), fetal-bovine serum, penicillin G and streptomycin sulphate were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-Tyrosine (disodium salt) was purchased from Research Organics Inc. (Cleveland, OH, U.S.A.). Pronase was from Calbiochem (San Diego, CA, U.S.A.). Carrier-free Na₂³⁵SO₄ and [³⁵S]methionine (1113 Ci/mmol) were from ICN Biomedicals (Irvine, CA, U.S.A.). ULTRAFREE-MC 5000 NMWL filter units and Centricon 10 microconcentrators (10000-molecular-mass cut-off) were purchased from Millipore Corp. (Bedford, MA, USA) and Amicon (Beverly, MA, U.S.A.) respectively. Chromatogram cellulose t.l.c. plates were from Eastman Kodak

Abbreviations used: TyrS, tyrosine O-sulphate; MEM, minimum essential medium; PAPS, 3'-phosphoadenosine 5'-phosphosulphate.

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Co. (New Haven, CT, U.S.A.). Chang liver cells (A.T.C.C. CCL 13), Caco-2 human intestinal epithelial cells (A.T.C.C. HTB 37), MDCK cells (A.T.C.C. CCL 34) and HepG2 human hepatoma cells (A.T.C.C. HB 8065) were all obtained from American Type Culture Collection (Rockville, MD, U.S.A.). All other chemicals were of the highest grades commercially available.

Cell culture

Chang liver cells, Caco-2 cells, MDCK cells and HepG2 cells were routinely maintained in MEM supplemented with 10 % fetal-bovine serum, penicillin G (30 µg/ml) and streptomycin sulphate (50 µg/ml), in a 5 % CO₂ atmosphere at 37 °C. Confluent cells, grown in individual wells of 24-well culture plates or 100 mm-diam. culture dishes, were used in the following experiments.

Metabolic labelling of cells with [³⁵S]sulphate or [³⁵S]methionine in the presence of different concentrations of cycloheximide

Confluent Chang liver cells, Caco-2 cells or MDCK cells grown in individual wells of a 24-well culture plate, preincubated in sulphate-free (prepared by omitting streptomycin sulphate and replacing MgSO₄ with MgCl₂) or methionine-deficient MEM for 12 h, were labelled with 0.25 ml portions of sulphate-free or low-methionine (25 % of the normal concentration) MEM containing [³⁵S]sulphate (0.3 mCi/ml) or [³⁵S]methionine (50 µCi/ml) and 0, 10, 20 or 30 µg/ml cycloheximide. At the end of a 20 h labelling, the media were collected for analysis of free Tyr[³⁵S] or [³⁵S]methionine-labelled proteins by the procedures described below.

Endocytotic degradation of tyrosine-[³⁵S]sulphated proteins by Chang liver cells incubated in media containing different concentrations of cycloheximide

Confluent Chang liver cells grown in individual wells of a 24-well culture plate, after a 6 h preincubation in MEM containing 0, 10, 20 or 30 µg/ml cycloheximide, were incubated in 0.25 ml portions of MEM containing approx. 750 000 c.p.m. of [³⁵S]sulphate-labelled proteins (prepared from the spent medium of [³⁵S]sulphate-labelled HepG2 human hepatoma cells as described below) and the same concentrations of cycloheximide. At 12, 24 and 36 h after the incubation, the media were collected for the analysis of free Tyr[³⁵S] and tyrosine-[³⁵S]sulphated proteins by the procedures described below.

Preparation of crude homogenates and the cytosolic and membrane fractions

Confluent Chang liver cells, Caco-2 cells or MDCK cells grown in five 100 mm dishes were scraped off, suspended in 10 ml of 20 mM Hepes/NaOH (pH 7.0) containing 250 mM sucrose, 30 µg/ml aprotinin and 1 mM phenylmethanesulphonyl fluoride, and homogenized in a Dounce tissue grinder. The crude homogenates prepared were centrifuged at 1000 g for 10 min, and the supernatants collected were ultracentrifuged at 100 000 g for 2 h. The pelleted membranes were washed with the same buffer solution and resuspended individually in 0.5 ml of 10 mM Hepes/NaOH (pH 7.0) containing 250 mM sucrose and 30 µg/ml aprotinin. The supernatants containing the cytosolic proteins were dialysed at 4 °C overnight against 10 mM Hepes/NaOH (pH 7.0) containing 250 mM sucrose. The crude homogenates, cytosolic fractions and membrane fractions prepared were analysed for the 'tyrosine sulphotransferase' activity by the assay procedure described below.

Enzymic-activity assay for the sulphation of L-tyrosine

To examine the enzymic activity catalysing the sulphation of tyrosine, the crude homogenate, cytosolic fraction or membrane fraction prepared from Chang liver cells, Caco-2 cells or MDCK cells was added to a reaction mixture containing, in a final volume of 100 µl, 50 mM Hepes buffer (pH 8.0), 0.25 M sucrose, 1 mM L-tyrosine, 10 mM MnCl₂, phosphatase inhibitors (25 mM NaF and 1 mM 5-AMP), protease inhibitors (30 µg/ml aprotinin, 30 µg/ml antipain, 300 µg/ml benzamidin and 30 µg/ml soybean trypsin inhibitor), and 4.5 µM of 3'-phospho-adenosine 5'-phospho[³⁵S]sulphate (PAP[³⁵S]) (15 Ci/mmol). The reaction was started by addition of 40 µl of enzyme preparation, allowed to proceed at 37 °C for 1 h, and terminated by heating at 100 °C for 3 min. The heated reaction mixture was centrifuged to clear the precipitates formed. The supernatant obtained was analysed for radiolabelled Tyr[³⁵S] by the two-dimensional thin-layer separation procedure described below. As a negative control, the cell homogenate was heated at 100 °C for 5 min before addition to the assay mixture.

Metabolic labelling of cells with [³⁵S]sulphate or [³⁵S]methionine in media containing different concentrations of L-tyrosine

Confluent Chang liver cells, Caco-2 cells or MDCK cells grown in individual wells of a 24-well culture plate, preincubated in sulphate- and tyrosine-deficient MEM for 12 h, were labelled with 0.25 ml portions of sulphate-free MEM containing [³⁵S]sulphate (0.3 mCi/ml) or [³⁵S]methionine (50 µCi/ml) and, respectively, 0.23, 1.15, 2.3 or 4.6 mM L-tyrosine. At the end of a 20 h labelling, the labelling media were collected for the analysis of free Tyr[³⁵S] or [³⁵S]methionine-labelled proteins.

Analysis of free Tyr[³⁵S] and [³⁵S]methionine-labelled proteins

For the analysis of free Tyr[³⁵S], 100 µl portions of the media prepared in the above-mentioned experiments were spin-filtered in ULTRAFREE-MC 5000 NMWL filter units. Samples (15 µl) of the filtrates obtained were each mixed with 1 µl of a 10 mg/ml synthetic TyrS solution, and spotted on to 20 cm × 20 cm cellulose t.l.c. plates for the analysis of Tyr[³⁵S] by a two-dimensional thin-layer separation procedure [19]. Briefly, the plates were first subjected to high-voltage electrophoresis (1000 V, 70 min) in 5 % (v/v) acetic acid/0.5 % (v/v) pyridine, pH 3.5. After electrophoresis, the plates were air-dried and subjected in the second dimension to ascending chromatography in butan-1-ol/88 % formic acid/propan-2-ol/water (3:1:1:1, by vol.). Upon completion of the chromatography, the plates were sprayed with ninhydrin solution (0.5 % in acetone). The ninhydrin-stained spots of TyrS were scraped off, suspended in 0.5 ml portion of water, and mixed with 4 ml portions of scintillation cocktail (Ecolume; ICN Radiochemicals), and counted for ³⁵S radioactivity. For the analysis of [³⁵S]methionine-labelled proteins, 50 µl samples of the media prepared in the above-mentioned experiments were mixed with equal amounts of a 2-fold concentrated Laemmli SDS sample buffer [20], heated for 3 min at 100 °C, and subjected to SDS/PAGE [20]. After electrophoresis, the gels were stained with Coomassie Blue, destained and dried. Gel-electrophoretic lanes corresponding to different samples were excised from the dried gels, sliced into small pieces, and placed in individual tubes. Then 4 ml of 50 mM NH₄HCO₃ solution containing 100 µg/ml Pronase was added to each tube, and the tubes were incubated for 24 h at 37 °C with constant shaking. Radiolabelled proteins fixed in the gel pieces were hydrolysed by Pronase and eluted from the gel. At the end of the incubation, 1 ml of 50 mM NH₄HCO₃ solution containing 0.4 mg/ml

Pronase was added to each tube, and the tubes were incubated for another 24 h with constant shaking. At the end of the second incubation, 0.5 ml samples of the Pronase hydrolysates were mixed with 4 ml portions of scintillation cocktail (Ecolume; ICN Radiochemicals), and counted for ^{35}S radioactivity.

Miscellaneous methods

TyrS was synthesized by the procedure developed by Jevons [21]. $\text{PAP}^{[35]\text{S}}$ (15 Ci/mmol) was synthesized from ATP and $^{[35]\text{S}}$ sulphate by using the sulphate-activating enzymes, ATP sulphurylase and adenosine 5'-phosphosulphate kinase, from *Bacillus stearothermophilus* as described previously [22]. SDS/PAGE was performed on 4.5–15% gradient polyacrylamide gels by the method of Laemmli [20]. Protein determination was based on the method of Lowry et al. [23] with bovine serum albumin as the standard. $^{[35]\text{S}}$ Sulphate-labelled proteins, used in the endocytotic degradation experiment described above, were prepared from the spent medium of $^{[35]\text{S}}$ sulphate-labelled HepG2 human hepatoma cells (A.T.C.C. HB 8065). Briefly, confluent HepG2 cells grown on a 100 mm culture dish, preincubated for 12 h in sulphate-free MEM, were labelled with 5 ml of sulphate-free MEM containing $^{[35]\text{S}}$ sulphate (0.3 mCi/ml). After labelling for 24 h, the spent medium collected was subjected to seven cycles of ultrafiltration in an Amicon Centricon 10 microconcentrator. After each cycle of ultrafiltration, 2 ml of PBS was added to the concentrated preparation (approx. 0.1 ml in volume). The $^{[35]\text{S}}$ sulphate-labelled protein preparation collected at the end of the seventh cycle was virtually free of low-molecular-mass $^{[35]\text{S}}$ sulphate-labelled compounds.

RESULTS AND DISCUSSION

As mentioned above, the functional relevance of the excretion of free TyrS in mammalian urine and the occurrence of the formation of free TyrS by sulphation *de novo* of L-tyrosine have remained virtually unknown for the past 40 years. We have attempted to search for clues to these unresolved issues by examining (i) the production and release of free TyrS by cells in which protein synthesis has been inhibited, (ii) the existence of

the enzymic activity catalysing the sulphation of L-tyrosine, and (iii) the dependence of the production and release of free TyrS on the L-tyrosine concentration present in the cell culture medium.

Generation of free TyrS in the presence of cycloheximide, a protein-synthesis inhibitor

We first examined the generation of free Tyr $^{[35]\text{S}}$ by cells labelled in the presence of cycloheximide, a potent protein-synthesis inhibitor [18]. The underlying assumption for this study is that, if free TyrS were formed exclusively through the turnover (degradation) of tyrosine-sulphated proteins, the generation of free TyrS should diminish in accord with the inhibition of protein synthesis. The continuous generation of free TyrS by cycloheximide-treated cells would be an indication that at least some of the free TyrS is formed directly by the sulphation of L-tyrosine. Chang liver cells, Caco-2 cells and MDCK cells were labelled with $^{[35]\text{S}}$ sulphate in the presence of different concentrations of cycloheximide for examining the generation of free Tyr $^{[35]\text{S}}$. As shown in Figure 1, each of the autoradiographs taken from the t.l.c. plates used for the analysis of the spent media of Chang liver cells, labelled in the presence of as much as 30 $\mu\text{g}/\text{ml}$ cycloheximide, revealed a radioactive spot coincident with the synthetic TyrS. The identity of this radioactive species being free Tyr $^{[35]\text{S}}$ was further indicated by (i) its co-crystallization with synthetic TyrS and (ii) the acid-lability of its bound sulphate (results not shown). It was also noted that the amount of free Tyr $^{[35]\text{S}}$ released into the medium by Chang liver cells increased linearly with time during the 20 h labelling period (results not shown). Similar findings were also observed for the spent media of Caco-2 cells and MDCK cells (not shown). Data on the quantitative determination of free Tyr $^{[35]\text{S}}$ generated by the three kinds of cells labelled in the presence of different concentrations of cycloheximide are compiled in Table 1. It was calculated that, for Chang liver cells, amounts equivalent to 95.3, 87.7 and 91.7% of the amount of free Tyr $^{[35]\text{S}}$ generated under normal conditions were detected in the spent media of the cells labelled in the presence of, respectively, 10, 20 and 30 $\mu\text{g}/\text{ml}$ cycloheximide. For Caco-2 cells, amounts equivalent to 41.1, 38.9 and 35.8% of the amount of free Tyr $^{[35]\text{S}}$ generated under

Figure 1 Generation of free Tyr $^{[35]\text{S}}$ by Chang liver cells labelled with $^{[35]\text{S}}$ sulphate in the presence of different concentrations of cycloheximide

The Figure shows the autoradiograph taken from the cellulose thin-layer plates used for the two-dimensional thin-layer analysis of the labelling media containing (a) 0, (b) 10, (c) 20 and (d) 30 $\mu\text{g}/\text{ml}$ cycloheximide. The dashed-line rings indicate the positions of synthetic TyrS standard detected by ninhydrin staining.

Table 1 Amounts of free Tyr^[35S] or [^{35S}]methionine-labelled proteins generated by Chang liver cells, Caco-2 cells and MDCK cells labelled with [^{35S}]sulphate or [^{35S}]methionine in the presence of different concentrations of cycloheximide

Data represent calculated mean values derived from three experiments.

Cell line	Cycloheximide concn. (μg/ml)	10 ⁻³ × Free Tyr ^[35S] released (c.p.m./mg of cell protein)	10 ⁻³ × [^{35S}]Methionine-labelled proteins secreted (c.p.m./mg of cell protein)
Chang liver	0	247 (100%)*	1890 (100%)†
	10	235 (95.1%)	206 (10.9%)
	20	217 (87.8%)	149 (7.9%)
	30	226 (91.5%)	143 (7.6%)
Caco-2	0	1221 (100%)*	1851 (100%)†
	10	502 (41.1%)	327 (17.7%)
	20	475 (38.9%)	279 (15.1%)
	30	437 (35.8%)	265 (14.3%)
MDCK	0	103 (100%)*	3732 (100%)†
	10	24 (23%)	264 (7.1%)
	20	25 (24%)	304 (8.1%)
	30	27 (26%)	166 (4.4%)

* % of the total amount of free Tyr^[35S] formed by cells labelled with [^{35S}]sulphate in the absence of cycloheximide.

† % of the total amount of total [^{35S}]methionine-labelled proteins produced and secreted by cells labelled with [^{35S}]methionine in the absence of cycloheximide.

normal conditions were detected. For MDCK cells, the calculated percentages were 23.2, 24.4 and 25.9%. Parallel experiments using [^{35S}]methionine for labelling revealed, in all three cases, a dramatic decrease in protein synthesis in cells labelled in the presence of cycloheximide (Table 1). It is noted that, although cycloheximide exerted between 89.1% and 92.4% inhibition of protein synthesis in Chang liver cells, the amounts of free Tyr^[35S] produced were still close to the level generated under normal conditions. For cycloheximide-treated Caco-2 and MDCK cells, less but yet significant amounts (35.8–41.1% and 23.2–25.9% of normal levels, respectively) of free Tyr^[35S] were still generated. These data could be interpreted as showing that, for these three kinds of cells maintained under normal conditions, different amounts of the free Tyr^[35S] might be generated by sulphation *de novo* of L-tyrosine, with the rest being derived from the turnover of tyrosine-[^{35S}]sulphated proteins. Alternatively, assuming that the degradation of tyrosine-sulphated proteins with release of TyrS does not occur, the decreased levels of free Tyr^[35S] detected in cycloheximide-treated cells might have been simply due to the decreased enzymic activity catalysing the sulphation of L-tyrosine. Arguing against the occurrence of sulphation *de novo* of L-tyrosine, however, it can also be speculated that the rate of turnover of the small amounts of tyrosine-[^{35S}]sulphated proteins produced by the cells treated with cycloheximide could have increased, resulting still in the generation of significant amounts of free Tyr^[35S]. Attempting to probe the third possibility, we have performed an endocytotic degradation experiment using Chang liver cells incubated in the presence of different concentrations of cycloheximide. Results obtained indicate that, on incubation for 12, 24 or 36 h, the cells incubated in media containing added [^{35S}]sulphate-labelled proteins plus 10, 20 or 30 μg/ml cycloheximide produced 31.3–50.5% of free Tyr^[35S] generated by cells incubated with added [^{35S}]sulphate-labelled proteins only (results not shown). These results suggest that, contrary to the speculated increase, the rate of endocytotic degradation of tyrosine-[^{35S}]sulphated

Figure 2 Enzymic sulphation of L-tyrosine using PAP^[35S] as the sulphate donor

The Figure shows the autoradiographs taken from the cellulose thin-layer plates used for the two-dimensional thin-layer analysis of the reaction mixture with Caco-2 cell homogenate as the source of tyrosine sulphotransferase. The dashed-line ring indicates the position of synthetic TyrS standard detected by ninhydrin staining.

proteins generating free Tyr^[35S] might have decreased instead in the cells incubated in the presence of cycloheximide.

Enzymic sulphation of L-tyrosine

To obtain direct evidence for the generation of free TyrS by the sulphation of L-tyrosine, radioactive PAP^[35S] was used as the sulphate donor to examine the presence of the enzymic activity catalysing the sulphation of L-tyrosine in the homogenates prepared from the three kinds of cell studied. As shown in Figure 2, the autoradiograph taken from the cellulose t.l.c. plate used for the analysis of the reaction mixture, with Caco-2 cell homogenate as the source of the enzyme, revealed indeed the production of Tyr^[35S]. (The identity of the radioactive species migrating in the opposite direction to TyrS appears to be tyramine O-[^{35S}]sulphate, on the basis of its co-migration with the synthetic tyramine O-sulphate.) Similar enzymic activities were also detected in the homogenates prepared from Chang liver cells and MDCK cells. We have tentatively designated the enzyme, which is responsible for the sulphation of L-tyrosine, as 'tyrosine sulphotransferase'. As shown in Table 2, the specific activities of the tyrosine sulphotransferase varied for the crude homogenates prepared from the three kinds of cells. These differences may reflect the different levels of the enzyme, and therefore the different capacities of the three kinds of cells to catalyse the sulphation of L-tyrosine. It is noted, however, that the specific activity of the crude homogenate prepared from Chang liver cells was considerably lower than that for the crude homogenate prepared from MDCK cells, even though Chang liver cells produced more free Tyr^[35S] than did MDCK cells during the metabolic labelling experiments (cf. Tables 1 and 3). Furthermore, a dramatic increase in the total activity of the tyrosine sulphotransferase was observed upon dialysis of the cytosolic fraction prepared from Chang liver cells. The reason for these discrepancies, which were consistently observed, is currently unknown. It remains to be determined to what extent the tyrosine sulphotransferase activity is responsible for the formation of free Tyr^[35S] in the three kinds of cells labelled under

Table 2 Activities of tyrosine sulphotransferase in crude homogenates, cytosolic fractions and membrane fractions prepared from Chang liver cells, Caco-2 cells and MDCK cells

Total activity is calculated based on the specific radioactivity of PAP[³⁵S] used in the enzymic assay and corrected for the total volume of the subcellular fraction prepared from the cells grown in five 100 mm tissue-culture dishes. Data represent calculated means $\pm$ S.D. derived from three experiments. Specific (Sp.) activity refers to activity per mg of protein present in the subcellular fraction.

Fraction	Chang liver cells		Caco-2 cells		MDCK cells	
	Total activity (fmol/min)	Sp. activity (fmol/min per mg)	Total activity (fmol/min)	Sp. activity (fmol/min per mg)	Total activity (fmol/min)	Sp. activity (fmol/min per mg)
Crude homogenate	90 $\pm$ 12	2.9	685 $\pm$ 66	49.0	160 $\pm$ 49	10.9
Cytosolic fraction	169 $\pm$ 49	17.6	639 $\pm$ 58	130	187 $\pm$ 53	50.3
Membrane fraction	2.1 $\pm$ 0.3	0.5	3.2 $\pm$ 0.3	0.8	1.8 $\pm$ 0.3	1.0

Table 3 Amounts of free Tyr[³⁵S] or [³⁵S]methionine-labelled proteins produced by Chang liver cells, Caco-2 cells and MDCK cells labelled with [³⁵S]sulphate or [³⁵S]methionine in media containing different concentrations of L-tyrosine

Data represent calculated mean values derived from three experiments.

Cell line	L-Tyrosine concn. (mM)	10 ⁻³ $\times$ Free Tyr[³⁵ S] released (c.p.m./mg of cell protein)	10 ⁻³ $\times$ [³⁵ S]Methionine-labelled proteins secreted (c.p.m./mg of cell protein)
Chang liver	0.23	323 (100%)*	2423 (100%)†
	1.15	608 (188%)	2383 (98.3%)
	2.30	796 (246%)	2236 (92.3%)
	4.60	832 (258%)	2195 (90.6%)
Caco-2	0.23	1436 (100%)*	2230 (100%)†
	1.15	2427 (169%)	2218 (99.5%)
	2.30	3012 (210%)	2337 (105%)
	4.60	4917 (342%)	2370 (106%)
MDCK	0.23	115 (100%)*	4306 (100%)†
	1.15	164 (143%)	4188 (97.3%)
	2.30	222 (193%)	3979 (92.4%)
	4.60	377 (329%)	3631 (84.3%)

* % of the total amount of free Tyr[³⁵S] formed by cells labelled with [³⁵S]sulphate in the medium containing 0.23 mM L-tyrosine.

† % of the total amount of total [³⁵S]methionine-labelled proteins produced and secreted by cells labelled with [³⁵S]methionine in the medium containing 0.23 mM L-tyrosine.

different conditions (cf. Tables 1 and 3). In terms of the subcellular distribution of the tyrosine sulphotransferase, most of the enzymic activity was found in the cytosolic fraction for all three kind of cells tested. The cytosolic location of the tyrosine sulphotransferase is similar to that found for many known aryl sulphotransferases [1,4]. Tyrosine sulphotransferase, however, is likely to be a new type of aryl sulphotranferase. Although some of the aryl sulphotransferases characterized previously have been shown to be capable of catalysing the sulphation of certain tyrosine derivatives [11,24,25], e.g. tyrosine methyl ester or tyrosine ethyl ester, none of them could use unmodified L-tyrosine as substrate. Considering that the role of the 'tyrosine sulphotransferase' presumably is to catalyse the sulphation of L-tyrosine, an amino acid needed for protein synthesis, to form TyrS, a waste compound to be excreted, it would be important

to investigate the mechanism(s) involved in regulating its enzymic activity *in vivo*.

An important issue that needs to be addressed is related to the functional relevance of the sulphation of L-tyrosine. It is known that, although L-tyrosine is essential for the biosynthesis of proteins, thyroid hormones, catecholamines, melanin etc., excess amounts of this amino acid, as found in various disease states related to tyrosinaemia, may have detrimental effects in afflicted individuals [26]. For eliminating the excess L-tyrosine, the catabolic pathways operating in mammals have been shown to include (i) deamination with the formation of *p*-hydroxyphenylpyruvic acid, which can be either converted into *p*-hydroxyphenylacetic acid and/or *p*-hydroxyphenyl-lactic acid, or further catabolized through homogentistic acid to form fumarate and acetoacetate, (ii) decarboxylation with the formation of *p*-tyramine, and (iii) *N*-acetylation with the formation of *N*-acetyltyrosine [26]. It is possible that sulphation may represent yet another mechanism for removing the excess L-tyrosine. In fact, considering that it involves a single reaction yielding a compound, TyrS, which is considerably more water-soluble than the other catabolic derivatives of L-tyrosine, sulphation may well be a more efficient way for removing excess L-tyrosine than are the above-mentioned catabolic pathways.

Dependence of the production of free TyrS on the L-tyrosine concentration in culture medium

As an attempt to probe the possible functional role of the sulphation of L-tyrosine as a means for removing excess L-tyrosine, we have examined the amounts of free Tyr[³⁵S] produced when Chang liver cells, Caco-2 cells and MDCK cells were labelled with [³⁵S]sulphate in media containing different concentrations of L-tyrosine. Data compiled in Table 3 showed that, indeed, when cells were incubated in media containing higher concentrations of L-tyrosine, there was a dramatic increase, proportional to the L-tyrosine concentration, in the amount of free Tyr[³⁵S] produced. Parallel experiments using [³⁵S]methionine for labelling revealed only minor changes in the rate of protein synthesis in cells labelled under these conditions. Also, neither did the amounts of tyrosine-[³⁵S]sulphated proteins synthesized show any significant changes with increasing concentration of L-tyrosine present in the culture media (results not shown). Taken together, the results obtained from the tyrosine-

dependence experiment may implicate sulphation as a means for removing excess L-tyrosine.

The finding of the production of free TyrS by the sulphation of L-tyrosine may have clinical implications. Since it appears to function for the removal of excess L-tyrosine, defects in the activity of the enzyme 'tyrosine sulphotransferase' could potentially lead to the elevation of the concentration of L-tyrosine *in vivo*, and therefore to the problems similar to those found in patients with tyrosinaemia. In this connection, it would be interesting to investigate whether some of the tyrosinaemia cases for which the etiology still remains unknown [26] might be due to the defective 'tyrosine sulphotransferase'. However, the fact that the malfunctioning of the enzymes involved in the catabolism of L-tyrosine [26], e.g. fumarylacetoacetate hydrolase (for tyrosinaemia I), tyrosine aminotransferase (for tyrosinaemia II), and *p*-hydroxyphenylpyruvate dioxygenase (for tyrosinaemia III) [27] still causes the elevated level of L-tyrosine in blood and urine may imply a limit in capacity for sulphation as a means for removing the excess L-tyrosine. It remains to be clarified therefore, how the sulphation and the different catabolic pathways work together to fine-tune the concentration of L-tyrosine in the body.

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