Sulphation by cultured cells

Cysteine, cysteinesulphinic acid and sulphite as sources for proteoglycan sulphate

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Bovine aortic smooth-muscle cells, bovine aortic endothelial cells, and IMR-90 human embryonic lung fibroblasts were tested to determine their ability to use cysteine or cysteine metabolites as a source of sulphate (SO₄). Cells were incubated in SO₄-depleted medium containing [³⁵S]cysteine, 0.3 mM-cysteinesulphinic acid or 0.3 mM-sulphite (SO₃). The [³⁵S]chondroitin sulphate produced by the different cells was found to vary considerably in degree of sulphation under these conditions. One line of smooth-muscle cells utilized cysteine effectively as a SO₄ source and thus produced chondroitin sulphate which was highly sulphated. IMR-90 fibroblasts produced partly sulphated chondroitin sulphate under these conditions, while another smooth-muscle cell line could not utilize cysteine, but could utilize cysteinesulphinic acid as a partial SO₄ source. In contrast with the above cells, endothelial cells could not use cysteine or cysteinesulphinic acid as a source of SO₄ and produced chondroitin with almost no SO₄. All of the cells were able to utilize SO₂. Incubation of the cells in the SO₄-depleted medium containing [³⁵S]cysteine confirmed that only the first line of smooth-muscle cells could convert significant amounts of [³⁵S]cysteine to [³⁵SO₄]. Furthermore, the addition of 0.4 mM inorganic SO₄ did not inhibit the production of SO₄ from cysteine by these cells.

INTRODUCTION

Sulphation is a wide-ranging process, occurring in numerous tissues, and involving many substances [1] such as proteoglycans, proteins, lipids, hormones and drugs. It is likely that sulphation is involved in many aspects of function, recognition and excretion, so that the control and degree of SO₄ substitution may have significant effects on an organism.

Although SO₄ may be found in various food substances, metabolism of S-containing amino acids is thought to provide the major supply of SO₄ in vivo [1]. Exogenous cysteine (or cystine), or cysteine formed from serine and the S of methionine, is oxidized to cysteine-sulphinate, which can then undergo either decarboxylation or transamination leading to taurine or pyruvate respectively, and SO₄. The presumed intermediate in the latter pathway is β-sulphinylpyruvate, which spontaneously decomposes to yield pyruvate and SO₄. The SO₄ is then oxidized to SO₃ by sulphite oxidase, an enzyme which is present in many tissues [2]. Studies with rat liver mitochondria [3], perfused rat liver [4], and hepatocytes [5] have indicated that inorganic SO₄ may also be produced by pathways that are independent of cysteinesulphinate. The percentage of cysteine S converted to taurine rather than to SO₄ varies substantially among species [6] and probably also varies considerably with cell type. With few exceptions, however, information concerning the formation of SO₄ from cysteine or methionine is only available for liver cells. Consequently, it is not known if other cells are capable of producing their own supply of SO₄ or are dependent on external sources of SO₄.

The major use of SO₄ by most cells is in the formation of proteoglycans. These proteoglycans can generally be labelled in cell cultures by adding ³⁵SO₄ to the growth medium. Thus the cells are apparently capable of obtaining their necessary SO₄ by direct uptake from extracellular fluids. The cellular uptake of SO₄ has been shown to be an active process and appears to vary somewhat with cell type [7,8]. In human IMR-90 lung fibroblasts, SO₄ uptake has been shown to occur mainly by SO₄/chloride exchange [7] and is independent of phosphate or Na transport. However, in hepatocytes, transport has been shown to occur by a Na-dependent route as well as a Na-independent route [8], and is also dependent on bicarbonate.

We have previously cultured bovine aortic endothelial cells [9] and human skin fibroblasts [10] in media containing substantial concentrations of cystine (cysteine) but low concentrations of SO₄. The cystine concentration of this medium is characteristic of many different tissue culture media. We found that the concentration of SO₄ needed for full sulphation of proteochondroitin approached the lower levels of SO₄ found in normal serum, indicating that cysteine was not utilized as a SO₄ source by these cells, and suggesting that extracellular sources of SO₄ might be limiting for

Abbreviations used: D O α-hyaluronate, 2-acetamido-2-deoxy-3-0-(D-glucuronic acid)-D-glucose; ADi-0S, 2-acetamido-2-deoxy-3-O-(β-D-Glc-4-enepyrinosyluronic acid)-D-galactose; ADi-4S, 2-acetamido-2-deoxy-3-O-(β-D-Glc-4-enepyrinosyluronic acid)-4-O-sulpho-D-galactose; ADi-6S, 2-acetamido-2-deoxy-3-O-(β-D-Glc-4-enepyrinosyluronic acid)-6-O-sulpho-D-galactose; PAPS, adenosine 3'-phosphohdenylphosphosulfate.

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sulphation of chondroitin by some cells *in vivo*. Under-sulphation of chondroitin in the presence of low levels of SO₄ has also been demonstrated in cartilage [11,12], suggesting that this tissue was incapable of utilizing cysteine as a SO₄ source. In contrast with these findings, a specific mutant line of Chinese hamster ovary (CHO) cells has been found to be capable of producing fully sulphated chondroitin [13] even though the cells lacked the ability to take up SO₄ from the medium. These cells were shown to utilize 0.2 mM-cysteine as their sole source of SO₄. Considering these variations and the importance of sulphation, a better understanding of SO₄ control by different cells and tissues would appear to be in order.

**MATERIALS AND METHODS**

Minimum Essential Medium with Earle’s salts (MEM), RPMI-1640 and SO₄-free RPMI-1640 medium, in which MgCl₂ was substituted for MgSO₄, were from Gibco (Grand Island, NY, U.S.A.) [3H]Glucosamine (42.5 Ci/mmol), [35S]Cysteine (1024 Ci/mmol), and liquid-scintillation counting mixture (Aquasure) were obtained from New England Nuclear (Boston, MA, U.S.A.). Chondroitin ABC lyase was purchased from Miles (Elkhart, IN, U.S.A.). Chondroitin 6-sulphate, chondroitin 4-sulphate, cysteinesulphinic acid, Na₂SO₃ (> 97 %), sulphite oxidase, and cytochrome c were from Sigma.

Bovine aortic endothelial cells and bovine aortic smooth-muscle cells were obtained from fresh calf aorta after collagenase treatment as described previously [14]. After the cultures were established, they were stored in complete medium containing 10% dimethyl sulphoxide in a liquid N₂ storage bank. IMR-90 human embryonic lung fibroblasts were originally obtained from American Type Culture Collection (Rockville, MD, U.S.A.). After removal from storage, the bovine aortic endothelial cells and smooth-muscle cells were grown to confluence [approx. (1.5–2) x 10⁶ cells] in six-well cluster dishes (Costar) with RPMI-1640 medium containing 20% fetal-calf serum. IMR-90 fibroblasts were grown to confluence [approx. (1–1.5) x 10⁶ cells] in MEM plus 10% fetal-calf serum. All cells were pre-incubated for 1 h in serum-free SO₄-free RPMI-1640 (which contains 0.2 mM-cysteine as a source of cysteine) before labelling in 1.5 ml of the SO₄-free RPMI-1640 to which [3H]glucosamine (12 x 10⁶ c.p.m./ml) was added. Cells were cultured in this medium, or in this medium with added 0.3 mM-cysteinesulphinic acid, or with added 0.3 mM-Na₂SO₄. After 5 h, portions (0.5 ml) of the medium were desalted on Sephadex G-50 equilibrated with 0.5 M-ammonium bicarbonate. The excluded fractions were pooled, freeze-dried, and re-dissolved in water (0.1 ml). Sulphation of [3H]chondroitin was monitored by digestion of portions (1000 c.p.m.) with chondroitin ABC lyase [15] (0.15 unit, 1 h, 37 °C) in a total volume of 0.02 ml followed by chromatography on Whatman No. 1 paper and analysed by high-voltage electrophoresis in 1 M-acetic acid/0.75 M-formic acid at 2000 V for 20 min. This provided a separation of [35S]cysteine from [3H]SO₄ and from other oxidation products. Detection was accomplished by overnight autoradiography with Kodak X-OMAT X-ray film, and by counting the eluted spots.

**RESULTS AND DISCUSSION**

The degree of sulphation of the [3H]chondroitin produced by bovine aortic endothelial cells, bovine aortic smooth-muscle cells, and human IMR-90 embryonic lung fibroblasts was examined by paper chromatographic analysis of chondroitin ABC lyase degradation products (Fig. 1). [3H]Glycoprotein and [3H]heparan sulphate remained at the origin and are not shown. [3H]-ΔHyalobiuronic acid from [3H]hyaluronic acid migrated faster and completely separate from ΔDi-0S (not shown). As shown in Fig. 1(a), degradation of [3H]-chondroitin produced by the early passage smooth-
Cysteine as a source of sulphate in cultured cells

... muscle cells incubated in SO₄-free medium resulted in mostly ΔDi-4S and ΔDi-6S and almost no ΔDi-0S. These results indicated that highly sulphated [³⁵S]cysteine sulphate was produced even though these cells were cultured in the absence of added SO₄. Thus, it appeared that these cells were capable of using cysteine (0.2 mM-cysteine) as a source of SO₄. The [³⁵S]cysteine produced by another higher passage smooth-muscle cell line (Fig. 1b) was less than 20% sulphated under the same conditions. Also shown in Fig. 1(b) is that these cells appeared to have some ability to utilize cysteinesulphinic acid. However, the chondroitin sulphate produced was only 60%, sulphated, indicating that cysteinesulphinic acid could only supply limited amounts of sulphate. Addition of 0.3 mM-Na₂SO₄ to the medium resulted in fully sulphated chondroitin sulphate, indicating that the cells contained sulphite oxidase. (Na₂SO₄ was analysed for possible contaminating SO₄ by the method of Ba precipitation followed by acidification [16]. Less than 2% SO₄ was found which agreed with the lot analysis of >97% SO₄ provided by Sigma.) When IMR-90 cells (Fig. 1c) were incubated in SO₄-free medium they produced partially sulphated chondroitin (approx. 70%). Addition of cysteinesulphinic acid did not increase the amount of chondroitin sulphate, while the addition of SO₄ resulted in the production of chondroitin sulphate which was essentially fully sulphated. Bovine aortic endothelial cells (Fig. 1d) incubated in SO₄-free medium containing cysteine or cysteinesulphinic acid produced [³⁵S]chondroitin with essentially no SO₄. Indeed, these cells require concentrations of SO₄ higher than 0.1 mM in order to produce fully sulphated chondroitin sulphate [9]. Another bovine endothelial cell line at an earlier passage yielded similar results (not shown). However, like the other cells, these endothelial cells were capable of utilizing SO₄ as a SO₄ source, suggesting that sulphite oxidase might be common to most, if not all, cells. A sulphite oxidase/cytochrome c assay (Sigma) of medium containing SO₄ was performed before and after a 20 h control incubation without cells. The results demonstrated that essentially equal amounts of SO₄ were present before and after the 20 h incubation. Thus, no appreciable amounts of SO₄ (certainly less than 0.1 mM) were spontaneously oxidized to SO₄, indicating that the production of SO₄ from SO₄ in the 5 h cell culture experiments was enzymic.

To confirm that cysteine was indeed the source of SO₄ in the SO₄-free RPMI medium, the early passage smooth-muscle cells and the endothelial cells were incubated in the SO₄-free RPMI containing [³⁵S]cysteine as well as [³⁵S]glucosamine. The bovine aortic endothelial cells did not utilize [³⁵S]cysteine as a SO₄ source, while the bovine aortic smooth-muscle cells did, even when 0.4 mM-SO₄ was present in the medium (Fig. 1d). With all cells, the [³⁵S]cysteine was incorporated effectively into ³⁵S-labelled protein, demonstrating that there were no deficiencies of [³⁵S]cysteine movement into the cells.

We next cultured several cell lines in RPMI-1640 medium containing [³⁵S]cysteine together with a physiological concentration (0.4 mM) of non-labelled SO₄ to see if the presence of SO₄ would affect the utilization of cysteine. Medium was spotted on Whatman No. 1 paper and subjected to high-voltage electrophoresis (Fig. 2, Table 1). As expected, the early-passage bovine aortic smooth-muscle cells converted much of the [³⁵S]cysteine to ³⁵SO₄ (lane 3), while little, if any, ³⁵SO₄ was produced by the bovine aortic endothelial cells (lane 5) or the IMR-90 fibroblasts (lane 6). Other ³⁵S label was found between the ³⁵SO₄ and ³⁵S]cysteine, representing intermediates which were not examined further. The addition of 0.4 mM inorganic SO₄ did not inhibit the oxidation of cysteine by the early-passage smooth-muscle cells (lane 4). (Similar results have been reported with CHO cells where high concentrations of SO₄ did not inhibit the

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**Table 1. Production of ³⁵SO₄ from ³⁵S]cysteine**

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>³⁵SO₄ (% of total ³⁵S)</th>
<th>0.4 mM-SO₄</th>
<th>No added SO₄</th>
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oxidation of cysteine to SO₄ [13].) Amounts of ³⁵SO₄ formed at various times are shown in Table 1, and indicate a considerable conversion. In agreement with these results, when 0.4 mM-SO₄ was present in the medium, the ³⁵SO₄ derived from [³⁵S]cysteine was diluted about 4-fold from a ³⁵S/³⁴S ratio of approx. 0.8 to one of approx. 0.2 (Fig. 1a). Furthermore, cells having a capability of utilizing cysteine would dilute added ³⁵SO₄ with SO₄ from their cysteine pool. Results would be particularly distorted if low ³⁵SO₄ concentrations were used. Thus failure to recognize a production of SO₄ from cysteine could lead to erroneous results in comparing the amounts of ³⁵SO₄ incorporated by cells under varying conditions.

Undersulphation of substances should also be expected to vary according to the required concentrations of intracellular SO₄ and/or adenosine 3'-phosphoadenylyl-phosphosulphate (PAPS) needed for a specific sulphotransferase. Thus a sulphotransferase with a low Kₘ for PAPS would be less affected under conditions of SO₄ deprivation than would a sulphotransferase with a higher Kₘ, such as chondroitin sulphotransferase [17]. For example, the Kₘ with regard to PAPS for phenol sulphotransferase, heparin N-sulphotransferase, heparan N-sulphotransferase and chondroitin sulphotransferase have been reported to be approx. 0.5 μM [18], 20 μM [19], 50 μM [20] and 500 μM [17], respectively. Significant undersulphation therefore would only occur in those compounds requiring high concentrations of PAPS for sulphation.

The results of this paper show that analysis of chondroitin sulphate provides a sensitive measure of SO₄ availability. Since most, if not all, cells produce chondroitin sulphate, this should prove useful in determining the capacity of cells to use cysteine, or other S-containing compounds as precursors of SO₄.

This research was supported by the Medical Research Service of the Veterans Administration and Grant AM-36984 from the National Institutes of Health.

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Received 30 December 1987/4 February 1988; accepted 24 February 1988