Sulphation by cultured cells

Cysteine, cysteinesulphinic acid and sulphite as sources for proteoglycan sulphate

Donald E. HUMPHRIES,*§ Cynthia K. SILBERT† and Jeremiah E. SILBERT†

*Connective Tissue Research Laboratory, Veterans Administration Outpatient Clinic, †Department of Medicine, and the ‡Veterans Administration Medical Center, Harvard Medical School, Boston, MA 02108, U.S.A.

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 (SO_4). Cells were incubated in SO_4 -depleted medium containing [^3H]glucosamine plus 0.2 mM-cystine, 0.3 mM-cysteinesulphinic acid or 0.3 mM-sulphite (SO_3). The [^3H]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 SO_4 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 SO_4 source. In contrast with the above cells, endothelial cells could not use cysteine or cysteinesulphinic acid as a source of SO_4 and produced chondroitin with almost no SO_4 . All of the cells were able to utilize SO_3 . Incubation of the cells in the SO_4 -depleted medium containing [^{35}S]cysteine confirmed that only the first line of smooth-muscle cells could convert significant amounts of [^{35}S]cysteine to $^{35}\text{SO}_4$. Furthermore, the addition of 0.4 mM inorganic SO_4 did not inhibit the production of SO_4 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 SO_4 substitution may have significant effects on an organism.

Although SO_4 may be found in various food substances, metabolism of S-containing amino acids is thought to provide the major supply of SO_4 *in vivo* [1]. Exogenous cysteine (or cystine), or cysteine formed from serine and the S of methionine, is oxidized to cysteinesulphinic acid, which can then undergo either decarboxylation or transamination leading to taurine or pyruvate respectively, and SO_4 . The presumed intermediate in the latter pathway is β -sulphinylpyruvate, which spontaneously decomposes to yield pyruvate and SO_3 . The SO_3 is then oxidized to SO_4 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 SO_4 may also be produced by pathways that are independent of cysteinesulphinic acid. The percentage of cysteine S converted to taurine rather than to SO_4 varies substantially among species [6] and probably also varies considerably with cell type. With few exceptions, however, information concerning the formation of SO_4 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 SO_4 or are dependent on external sources of SO_4 .

The major use of SO_4 by most cells is in the formation of proteoglycans. These proteoglycans can generally be labelled in cell cultures by adding $^{35}\text{SO}_4$ to the growth medium. Thus the cells are apparently capable of obtaining their necessary SO_4 by direct uptake from extracellular fluids. The cellular uptake of SO_4 has been shown to be an active process and appears to vary somewhat with cell type [7,8]. In human IMR-90 lung fibroblasts, SO_4 uptake has been shown to occur mainly by SO_4 /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 SO_4 . The cystine concentration of this medium is characteristic of many different tissue culture media. We found that the concentration of SO_4 needed for full sulphation of proteoglycans approached the lower levels of SO_4 found in normal serum, indicating that cysteine was not utilized as a SO_4 source by these cells, and suggesting that extracellular sources of SO_4 might be limiting for

Abbreviations used: $\Delta\text{hyalobiuronic acid}$, 2-acetamido-2-deoxy-3- O -(β -D-Glc-4-enepyranosyluronic acid)-D-glucose; $\Delta\text{Di-0S}$, 2-acetamido-2-deoxy-3- O -(β -D-Glc-4-enepyranosyluronic acid)-D-galactose; $\Delta\text{Di-4S}$, 2-acetamido-2-deoxy-3- O -(β -D-Glc-4-enepyranosyluronic acid)-4- O -sulpho-D-galactose; $\Delta\text{Di-6S}$, 2-acetamido-2-deoxy-3- O -(β -D-Glc-4-enepyranosyluronic acid)-6- O -sulpho-D-galactose; PAPS, adenosine 3'-phosphoadenylyl-phosphosulphate.

§ To whom correspondence and reprint requests should be addressed: Veterans Administration Outpatient Clinic, 17 Court Street, Boston, MA 02108, U.S.A.

sulphation of chondroitin by some cells *in vivo*. Under-sulphation of chondroitin in the presence of low levels of SO_4 has also been demonstrated in cartilage [11,12], suggesting that this tissue was incapable of utilizing cysteine as a SO_4 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 SO_4 from the medium. These cells were shown to utilize 0.2 mM-cysteine as their sole source of SO_4 . Considering these variations and the importance of sulphation, a better understanding of SO_4 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 SO_4 -free RPMI-1640 medium, in which MgCl_2 was substituted for MgSO_4 , were from GIBCO (Grand Island, NY, U.S.A.) [^3H]Glucosamine (42.5 Ci/mmol), [^{35}S]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, cysteinesulphonic acid, Na_2SO_3 (> 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 sulfoxide in a liquid N_2 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. $(1.5-2) \times 10^6$ cells] in six-well cluster dishes (Costar) with RPMI-1640 medium containing 20% fetal-calf serum. IMR-90 fibroblasts were grown to confluence [approx. $(1-1.5) \times 10^6$ cells] in MEM plus 10% fetal-calf serum. All cells were pre-incubated for 1 h in serum-free SO_4 -free RPMI-1640 (which contains 0.2 mM-cystine as a source of cysteine) before labelling in 1.5 ml of the SO_4 -free RPMI-1640 to which [^3H]glucosamine (12×10^6 c.p.m./ml) was added. Cells were cultured in this medium, or in this medium with added 0.3 mM-cysteinesulphonic acid, or with added 0.3 mM- Na_2SO_3 . 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 with a descending system of 1-butanol/acetic acid/1 M-ammonium hydroxide (2:3:1, by vol.) [15]. All degradations contained 0.15 mg each of chondroitin, chondroitin 4-sulphate and chondroitin 6-sulphate, which yielded $\Delta\text{Di-OS}$, $\Delta\text{Di-4S}$ and $\Delta\text{Di-6S}$ after degradation, respectively. These unsaturated disaccharides were localized under u.v. light, eluted from the paper with water, and radioactivity was determined by liquid-scintillation spectrometry.

Fig. 1. Paper chromatography of disaccharides formed by chondroitin ABC lyase degradations of [^3H]chondroitin $^{35}\text{SO}_4$ or [^3H]chondroitin sulphate

[^3H]Glucosamine-labelled glycosaminoglycans formed by cells incubated in SO_4 -depleted medium containing [^{35}S]cysteine, cystine, or cystine plus cysteine metabolites or in SO_4 -containing medium, were mixed with chondroitin, chondroitin 4-sulphate, and chondroitin 6-sulphate and degraded with chondroitin ABC lyase before paper chromatography in 1-butanol/acetic acid/1 M-ammonium acetate (2:3:1, by vol.) Strips of 1 cm were eluted and analysed for radioactivity by scintillation counting. Migration of the degradation products $\Delta\text{Di-6S}$, $\Delta\text{Di-4S}$ and $\Delta\text{Di-OS}$ is shown at the top. Migration is shown of the degradation products of [^3H]chondroitin $^{35}\text{SO}_4$ or [^3H]chondroitin sulphate formed by (a) an early-passage smooth-muscle cell line, (b) a later-passage smooth-muscle cell line, (c) IMR-90 fibroblasts, and (d) endothelial cells.

RPMI medium from cells incubated for various time intervals with added [^{35}S]cysteine was spotted 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 [^{35}S]cysteine from $^{35}\text{SO}_4$ 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]- $\Delta\text{Hyalobiuronic acid}$ from [^3H]hyaluronic acid migrated faster and completely separate from $\Delta\text{Di-OS}$ (not shown). As shown in Fig. 1(a), degradation of [^3H]chondroitin produced by the early passage smooth-

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 [³H]chondroitin 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-cystine) as a source of SO₄. The [³H]chondroitin produced by another higher-passage smooth-muscle cell line (Fig. 1*b*) 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-Na₂SO₃ to the medium resulted in fully sulphated chondroitin sulphate, indicating that the cells contained sulphite oxidase. (Na₂SO₃ 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% SO₃ provided by Sigma.) When IMR-90 cells (Fig. 1*c*) 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 SO₃ resulted in the production of chondroitin sulphate which was essentially fully sulphated. Bovine aortic endothelial cells (Fig. 1*d*) incubated in SO₄-free medium containing cysteine or cysteinesulphinic acid produced [³H]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 SO₃ 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 SO₃ was performed before and after a 20 h control incubation without cells. The results demonstrated that essentially equal amounts of SO₃ were present before and after the 20 h incubation. Thus, no appreciable amounts of SO₃ (certainly less than 0.1 mM) were spontaneously oxidized to SO₄, indicating that the production of SO₄ from SO₃ 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 [³H]glucosamine. The bovine aortic endothelial cells did not utilize [³⁵S]cysteine as a ³⁵SO₄ source, while the bovine aortic smooth-muscle cells did, even when 0.4 mM-SO₄ was present in the medium (Fig. 1*a*). 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

Fig. 2. High-voltage electrophoresis of medium following incubation of cells with [³⁵S]cysteine

Cells were incubated in medium containing [³⁵S]cysteine for 5 h. Portions were then electrophoresed and visualized as described in the Materials and methods section. Lane 1, [³⁵S]cysteine and ³⁵SO₄ standards; lane 2, [³⁵S]cysteine standard; lane 3, smooth-muscle cells incubated with [³⁵S]cysteine and no SO₄; lane 4, smooth-muscle cells incubated with [³⁵S]cysteine and 0.4 mM-SO₄; lane 5, endothelial cells incubated with [³⁵S]cysteine and no SO₄; lane 6, fibroblasts incubated with [³⁵S]cysteine and no SO₄.

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

Incubation time (h)	³⁵ SO ₄ (% of total ³⁵ S)	
	0.4 mM-SO ₄	No added SO ₄
0	—	1
1	3	3
2	5	5
5	14	15

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

oxidation of cysteine to SO_4 [13].) Amounts of $^{35}\text{SO}_4$ formed at various times are shown in Table 1, and indicate a considerable conversion. In agreement with these results, when 0.4 mM-SO_4 was present in the medium, the $^{35}\text{SO}_4$ derived from [^{35}S]cysteine was diluted about 4-fold from a $^{35}\text{S}/^3\text{H}$ ratio of approx. 0.8 to one of approx. 0.2 (Fig. 1a). Furthermore, cells having a capability of utilizing cysteine would dilute added $^{35}\text{SO}_4$ with SO_4 from their cysteine pool. Results would be particularly distorted if low $^{35}\text{SO}_4$ concentrations were used. Thus failure to recognize a production of SO_4 from cysteine could lead to erroneous results in comparing the amounts of $^{35}\text{SO}_4$ incorporated by cells under varying conditions.

Undersulphation of substances should also be expected to vary according to the required concentrations of intracellular SO_4 and/or adenosine 3'-phosphoadenylyl-phosphosulphate (PAPS) needed for a specific sulphotransferase. Thus a sulphotransferase with a low K_m for PAPS would be less affected under conditions of SO_4 deprivation than would a sulphotransferase with a higher K_m , such as chondroitin sulphotransferase [17]. For example, the K_m with regard to PAPS for phenol sulphotransferase, heparin *N*-sulphotransferase, heparan *N*-sulphotransferase and chondroitin sulphotransferase have been reported to be approx. $0.5\text{ }\mu\text{M}$ [18], $20\text{ }\mu\text{M}$ [19], $50\text{ }\mu\text{M}$ [20] and $500\text{ }\mu\text{M}$ [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 SO_4 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 SO_4 .

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