Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 7160–7164, November 1985
Biochemistry

Tyrosine sulfation of proteins from the human hepatoma cell line HepG2

(fibrinogen/α-fetoprotein/fibronectin)

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Contributed by Fritz Lipmann, June 7 1985

ABSTRACT **[^{35}S]Sulfate labeling of the human hepatoma cell line HepG2 showed it to contain many sulfated proteins of diverse molecular weight range. The isolation of tyrosine *O*-sulfate indicated the supernatant fraction to contain a 5- to 7-fold higher level than the cellular fraction at the end of a 24-hr incubation. The proteins in the supernatant fraction were immunoprecipitated and examined for sulfation. Of 15 proteins tested, 7 were found to be sulfated as indicated by [^{35}S]sulfate incorporation into proteins separated by NaDodSO$_4$/PAGE and detected by autoradiography. The ^{35}S-labeled bands were excised from the dried gel and subjected to extensive Pronase hydrolysis and the hydrolysates were analyzed for tyrosine [^{35}S]sulfate by a two-dimensional procedure combining high-voltage electrophoresis and thin-layer chromatography [Liu, M. C. & Lipmann, F. (1984) *Proc. Natl. Acad. Sci. USA* 81, 3695–3698]. Of the sulfated proteins, three—fibrinogen, α-fetoprotein, and fibronectin—were found to contain tyrosine *O*-sulfate. The simultaneous presence of carbohydrate-bound sulfate, however, could not be exactly determined, but the other four [^{35}S]sulfate-containing proteins—α$_1$-antitrypsin, α$_1$-antichymotrypsin, α$_2$-macroglobulin, and transferrin—did not reveal any tyrosine *O*-sulfate and might be sulfated on their carbohydrate moieties.**

The covalent modification of proteins by sulfation may occur in two ways: (*i*) through the linkage to the carbohydrate moiety of glycoproteins and (*ii*) on tyrosine residues in the polypeptide backbone. Examples of the former type of sulfation include a large number of glycoproteins. They are found, for example, in developing sea urchin embryos (1), the contact site A glycoproteins of *Dictyostelium discoideum* (2), some virus-induced sulfated glycoproteins (3, 4), and the newly characterized neural cell adhesive protein, N-CAM (neural cell-adhesion molecule) (5). All these proteins have been suggested to be involved in the developmental processes. Direct sulfation (so far found exclusively on tyrosine), on the other hand, represents only a small fraction of the total sulfate bound.

Although first discovered in bovine fibronogen >30 years ago (6), the relatively widespread occurrence of protein tyrosine sulfation was not recognized until the study using a [^{35}S]sulfate radioactive-labeling technique by Huttner (7). In a previous study (8), we compared protein tyrosine sulfation in normal and avian sarcoma virus-transformed rat embryo fibroblasts, and we later isolated tyrosine *O*-sulfate [Tyr(SO$_3$)] from fibronectin (9). Furthermore, proteins so far identified to be tyrosine sulfated include complement C4 (10), two pairs of proteins from rat pheochromocytoma cells (11), and a hybridoma immunoglobulin G (12). To further reveal the identities of other tyrosine-sulfated proteins, we decided to use a human hepatoma cell line, HepG2, because this cell line is known to be capable of producing many plasma proteins for which there are antisera commercially available. In this case, we could not compare malignantly transformed and normal cells because the normal human hepatocytes were not available. We plan to carry out such a comparison by using rat tissue where both normal and transformed cells appear to be available.

In this work, we examined the overall sulfation of HepG2 proteins. The level of protein tyrosine sulfation in both the cellular and culture supernatant fractions were measured. Specific proteins in the supernatant fraction were immunoprecipitated and examined with respect to sulfation. Of the 15 proteins tested, on electrophoresis, 7 revealed ^{35}S radioactive bands, as detected by autoradiography. Further analysis showed that 3 of them—fibrinogen, α-fetoprotein and fibronectin—contained Tyr(SO$_3$).

MATERIALS AND METHODS

Materials. Protein molecular weight standards were obtained from Bethesda Research Laboratories. Gelatin-Sepharose 4B and protein A-Sepharose CL-4B were products of Pharmacia. Pronase and antisera against human α$_1$-antitrypsin, apolipoprotein A, apolipoprotein B, α$_1$-antichymotrypsin, ceruloplasmin, C-reactive protein, fibronectin, and plasminogen were from Calbiochem–Behring. Purified IgG preparations against human α$_2$-macroglobulin, complement C3c, α-fetoprotein, albumin, fibrinogen, transferrin, hepatoglobin, and whole plasma were from DAKO (Westbury, NY). Tyr(SO$_3$) standard was synthesized according to the procedure of Jevons (13). Carrier-free [^{35}S]sulfuric acid was from New England Nuclear. Precoated TLC cellulose plates were purchased from Brinkmann. All other reagents were of the highest grade commercially available.

Cell Culture and Radioactive Labeling. Human hepatoma-derived cell line HepG2 was routinely grown in minimal essential medium (ME medium) supplemented with 10% fetal calf serum (GIBCO). Subconfluent cells were labeled with [^{35}S]sulfate (0.3 mCi/ml; 1 Ci = 37 GBq) in sulfate-free (prepared by replacing MgSO$_4$ with MgCl$_2$) ME medium without serum. For the experiments on fibronectin, 10% dialyzed fibronectin-free (gelatin-Sepharose-treated) calf serum was incorporated for reasons described below. Labeling was for 24 hr. The culture supernatant was then removed and three protease inhibitors—Trasylol (200 units/ml), L-1-tosylamido-2-phenylethyl chloromethyl ketone (0.1 mM), and phenylmethylsulfonyl fluoride (1 mM)—were immediately added to prevent protein degradation by endogenous proteases. Cells were rinsed three times with ME medium and lysed in Laemmli sample buffer (14) for further workup.

Immunoprecipitation. For immunoprecipitation, an aliquot of labeled culture supernatant was incubated in phosphate-buffered saline with each specific antiserum (or purified IgG

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Abbreviation: Tyr(SO$_3$), tyrosine-*O*-sulfate.

preparation) for 1 hr at 0°C. Protein A-Sepharose CL-4B was then added and the mixture was shaken at 4°C for 30 min. Protein A-Sepharose bound with the immune complex was brought down by centrifugation, washed five times with phosphate-buffered saline, and placed in Laemmli sample buffer (14) for subsequent $NaDodSO_4$/polyacrylamide gel electrophoresis.

Tyr(SO_3) Analysis. Dialyzed concentrated culture supernatant, cell lysate, and immunoprecipitated fractions were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis on a 5.5–16% polyacrylamide gradient gel according to the method of Laemmli (14). After electrophoresis, the gel was stained with Coomassie brilliant blue in 50% methanol/10% acetic acid, and destained with 25% methanol/7.5% acetic acid solution. After destaining, the gel was dried under vacuum at room temperature and autoradiographed. For the measurement of Tyr(SO_3) levels in the cell lysate and culture supernatant fractions, the complete electrophoretic lane was cut from the top to the dye front into eight gel pieces at positions of molecular weight standards used: cytochrome *c* (M_r, 12,300), β-lactoglobulin (M_r, 18,400), α-chymotrypsinogen (M_r, 25,700), ovalbumin (M_r, 43,000), bovine serum albumin (M_r, 68,000), phosphorylase b (M_r, 92,500), and myosin (H chain) (M_r, 200,000). For the examination of individual proteins, the radioactive bands corresponding to the immunoprecipitated proteins were located by autoradiography and excised from the gel. Proteins in the excised gel pieces were then eluted and hydrolyzed by Pronase. Hydrolysates were analyzed for Tyr(SO_3) as described (8).

RESULTS

Sulfation Pattern and Tyr(SO_3) Content of HepG2 Proteins in Cellular and Culture Supernatant Fractions. Fig. 1 shows the $NaDodSO_4$ gel electrophoresis pattern of HepG2 proteins in the cellular and culture supernatant fractions and the corresponding autoradiograph. Although ^{35}S radioactive bands appeared throughout the entire molecular weight range in both fractions, sulfation (as indicated by the distinct radioactive bands) was found to be more prevalent among proteins (or their subunits) of M_r 43,000–68,000. The smearing background spreading over the entire electrophoretic lane is due to [^{35}S]sulfated proteoglycans concurrently synthesized by HepG2 cells. Fig. 2 shows the Tyr(SO_3) contents of HepG2 proteins in different molecular weight regions. On the per mg protein basis, Tyr(SO_3) level of proteins in the culture supernatant was 5- to 7-fold higher than that of the cellular proteins over the entire molecular weight range. It is to be noted that ^{35}S radioactivity of Tyr($^{35}SO_3$) constituted only a small portion (0.3–4%) of the total ^{35}S radioactivity present in each gel piece (data not shown). The majority of [^{35}S]sulfate, therefore was carbohydrate-bound. However, because of the interference from [^{35}S]sulfated proteoglycans (whose sulfate is carbohydrate-bound), sulfation on the carbohydrate moiety of glycoproteins is difficult to evaluate.

Preliminary Screening of Sulfated HepG2 Proteins. Fifteen proteins released into the culture supernatant by HepG2 cells were immunoprecipitated by using either antisera or purified IgG preparations and subjected to $NaDodSO_4$ gel electrophoresis. Fig. 3 shows the autoradiograph of immunoprecipitated proteins after electrophoresis. Contamination by [^{35}S]sulfated proteoglycans occurred in all cases of immunoprecipitation, as suggested by the smearing background along the electrophoretic lane. A nonspecific radioactive band (M_r, 74,000) of unknown identity was also found in each case and, therefore, is probably not part of the specific protein immunoprecipitated. Other than this nonspecific band and the hazy background due to [^{35}S]sulfated proteoglycans, 7 of the 15 proteins examined were found to exhibit ^{35}S radioactive bands at positions corresponding to their subunit molecular weights. Those proteins found to be sulfated are the following: α_1-antitrypsin (M_r, 52,000), α_1-antichymotrypsin (M_r, 65,000), α_2-macroglobulin (M_r, 180,000), α-fetoprotein (M_r, 68,000), fibrinogen (M_r of Bβ subunit, 55,000), transferrin

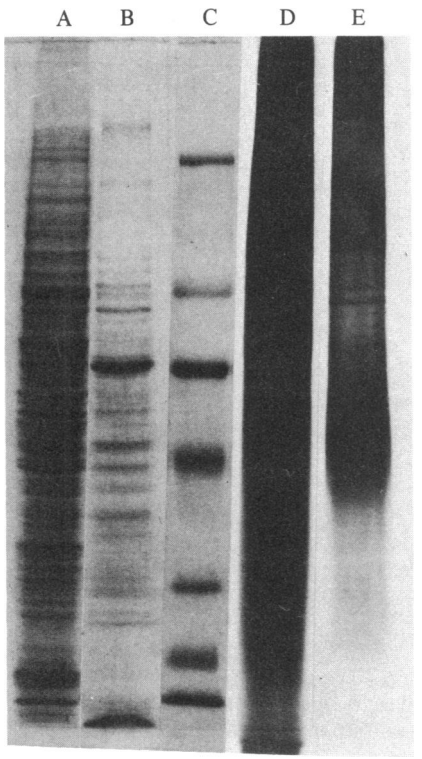

FIG. 1. $NaDodSO_4$ gel electrophoretic patterns of [^{35}S]sulfate-labeled HepG2 proteins in cellular (lane A) and culture supernatant (lane B) fractions. Lanes D and E show the corresponding autoradiographs of lanes A and B, respectively. Protein standards shown in lane C are cytochrome *c* (M_r, 12,300), β-lactoglobulin (M_r, 18,400), α-chymotrypsinogen (M_r, 25,700), ovalbumin (M_r, 43,000), bovine serum albumin (M_r, 68,000), phosphorylase b (M_r, 92,500), and myosin (H chain) (M_r, 200,000).

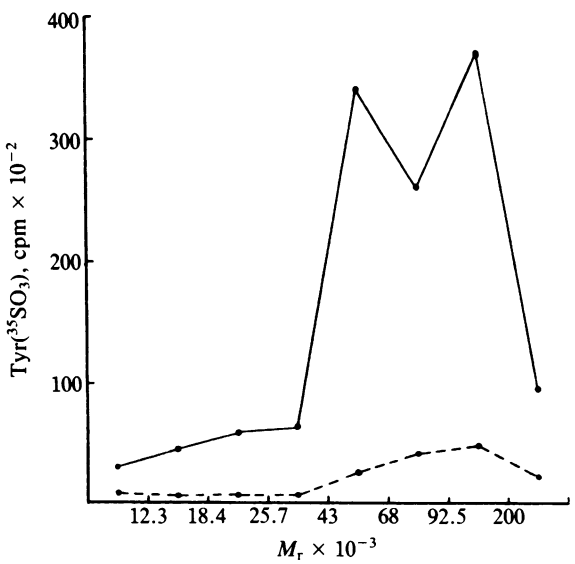

FIG. 2. Tyr(SO_3) contents of cellular (●---●) and culture supernatant (●—●) proteins of HepG2 cells in different molecular weight regions. Estimation of the Tyr(SO_3) content was based on 1 mg of cellular or culture supernatant proteins used for $NaDodSO_4$ gel electrophoresis.

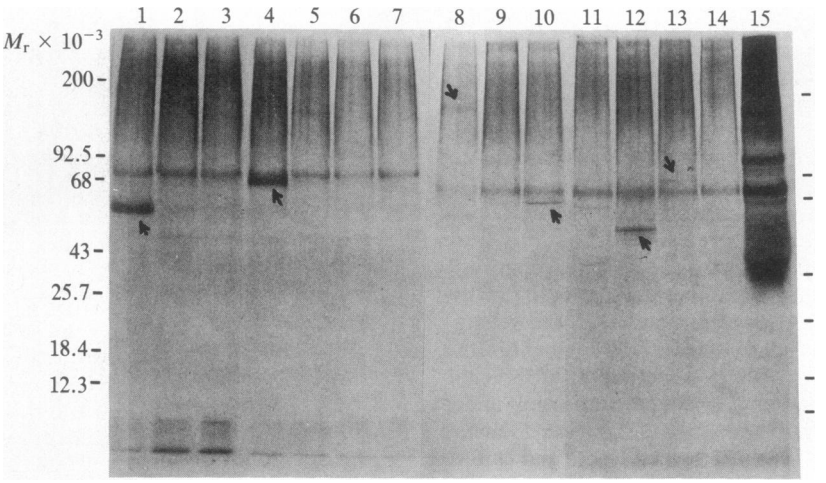

FIG. 3. Autoradiograph of immunoprecipitated HepG2 culture supernatant proteins after NaDodSO$_4$ gel electrophoresis. [^{35}S]Sulfate-labeled proteins immunoprecipitated are as follows: lane 1, α_1-antitrypsin; lane 2, apolipoprotein A; lane 3, apolipoprotein B; lane 4, α_1-antichymotrypsin; lane 5, ceruloplasmin; lane 6, C-reactive protein; lane 7, plasminogen; lane 8, α_2-macroglobulin; lane 9, complement C3c; lane 10, α-fetoprotein; lane 11, albumin; lane 12, fibrinogen; lane 13, transferrin; lane 14, hepatoglobin; lane 15, whole plasma proteins. ^{35}S radioactive protein bands corresponding to the six sulfated proteins immunoprecipitated (the case of fibronectin is illustrated in Fig. 4) are indicated by the arrows.

(M_r, 80,000), and fibronectin (M_r, 220,000). The radioactive band of the immunoprecipitated fibronectin, although faintly visible, was overwhelmed by the contaminating [^{35}S]sulfated proteoglycans (not shown). We have previously found that by incorporating fibronectin-free calf serum (which is also rich in proteoglycans) into the labeling medium, the contamination by [^{35}S]sulfated proteoglycans simultaneously synthesized could be greatly reduced (11). Taking advantage of this fact and the high affinity of fibronectin for gelatin-Sepharose, we were able to isolate [^{35}S]sulfate-labeled fibronectin that was only slightly contaminated with [^{35}S]sulfated proteoglycans. As shown in Fig. 4, the isolated fibronectin exhibited a clear radioactive band at the position of M_r ≈220,000 under reducing conditions (with 2-mercaptoethanol); under nonreducing conditions (without 2-mercaptoethanol), a major radioactive band with slower electrophoretic mobility and a minor one at the position of M_r ≈220,000 were detected. These results were typical of a dimeric fibronectin previously found for the secreted fibronectin from rat embryo fibroblasts (9).

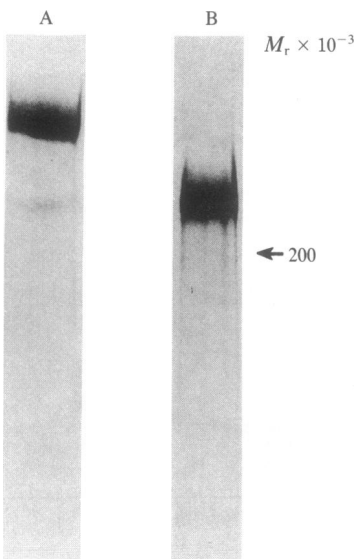

FIG. 4. Autoradiograph of gelatin-Sepharose-purified [^{35}S]sulfate-labeled fibronectin electrophoresed in a 4.5–10% polyacrylamide gradient gel under nonreducing (lane A) and reducing (lane B) conditions.

Tyr(SO$_3$) Analysis of Sulfated HepG2 Proteins. Radioactive bands of the seven sulfated proteins, located by autoradiograph, were excised from the dried gel and individually analyzed for Tyr(SO$_3$). Fig. 5 shows the autoradiographs of Pronase hydrolysates of fibrinogen, α-fetoprotein, and fibronectin after the two-dimensional separation. In all cases, the ninhydrin spot of Tyr(SO$_3$) as marked by the broken line was found to be radioactive. These results indicate that the three proteins were tyrosine-sulfated. The trailing radioactive lane from the origin along the first-dimensional separation (high-voltage thin-layer electrophoresis) also found in each case is probably derived from the carbohydrate-bound [^{35}S]sulfate (8). Due to the contamination by [^{35}S]sulfated proteoglycans, no conclusion can be drawn concerning the presence of [^{35}S]sulfate on the carbohydrate moiety of these three glycoproteins. Aside from the trailing radioactive lane, two additional radioactive spots of unknown identity were present. They are not likely due to other sulfated amino acids such as serine *O*-sulfate and threonine *O*-sulfate, because these compounds migrate much faster than Tyr(SO$_3$) during high-voltage electrophoresis.

The other four sulfated proteins—α_1-antitrypsin, α_1-antichymotrypsin, α_2-macroglobulin, and transferrin—displayed only trailing radioactive lanes along the first-dimensional analysis (not shown) when their Pronase hydrolysates were similarly analyzed. Therefore, these proteins are not tyrosine-sulfated, but rather appear to be sulfated only on their carbohydrate moieties.

DISCUSSION

In this study, we examined the sulfation of cellular and culture supernatant proteins from a human hepatoma cell line, HepG2. Radioactive labeling with [^{35}S]sulfate revealed that many of them were sulfated. Further analysis showed that Tyr(SO$_3$) was present in the Pronase hydrolysate of proteins throughout the entire molecular weight range. These results provided further evidence on tyrosine sulfation being a widespread protein modification. The apparent enrichment of tyrosine sulfation among culture supernatant proteins as compared to cellular proteins deserves special attention. Linkage of this finding with the recent hypothesis by Baeuerle and Huttner (12) that tyrosine sulfation serves as a

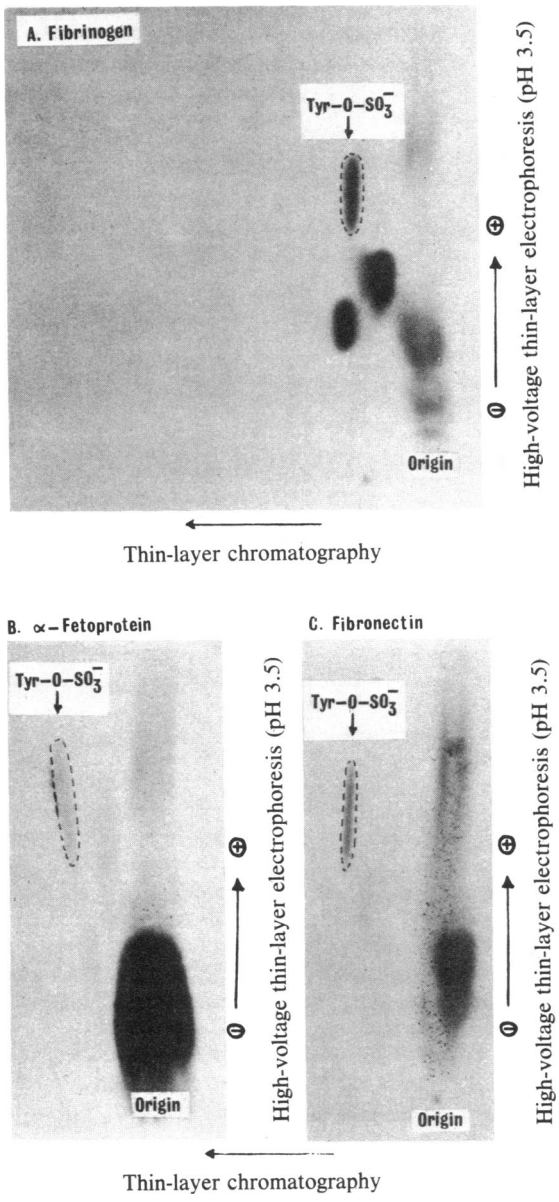

FIG. 5. Autoradiograph of Pronase hydrolysates of [^{35}S]sulfate-labeled fibrinogen (*A*), α-fetoprotein (*B*), and fibronectin (*C*) after two-dimensional analysis. Broken line indicates the ninhydrin spot of Tyr(SO_3).

signal for protein secretion needs to be clarified. Immunoprecipitation of 15 proteins released into the culture supernatant by HepG2 cells using antisera or purified IgG preparations, followed by NaDodSO$_4$ gel electrophoresis, revealed that 7 of them were sulfated as indicated by their ^{35}S radioactive protein bands. Three of the 7 sulfated proteins—fibrinogen, α-fetoprotein, and fibronectin—were found to contain Tyr(SO_3). The presence of carbohydrate-bound sulfate in them was, however, not clear because of the interference from the contaminating sulfated proteoglycans. The other four sulfated proteins—α_1-antitrypsin, α_1-antichymotrypsin, α_2-macroglobulin, and transferrin—did not exhibit any tyrosine-linked sulfate and, therefore, are thought to be sulfated on their carbohydrate moieties.

Although tyrosine sulfation occurs in fibrinopeptide B split from fibrinogen in many mammalian species, human fibrinopeptide B does not contain this sulfated amino acid (15). The presence of Tyr(SO_3) in human fibrinogen was nevertheless suggested by the evidence from spectroscopic analysis on alkaline hydrolysate of human fibrin (13). Quantitative results showed that approximately 2 mol of Tyr(SO_3) were present per mol of fibrin. Our data confirmed the previous finding and further indicated the location of Tyr(SO_3) being in the Bβ subunit of human fibrinogen molecules. Since fibrinopeptide B is derived from the NH_2-terminal end of the Bβ subunit, Tyr(SO_3) in human fibrinogen must locate at a position more distal from the NH_2 terminus. In view of the different locations of Tyr(SO_3) in human and bovine (or other species) fibrinogen molecules, it will be interesting to investigate the structural–functional relationship of tyrosine sulfation in the overall blood coagulation process.

α-Fetoprotein is known to be an embryonal protein, being present only transiently in fetal serum (16). The same protein appears in the sera of patients with hepatoma or other liver diseases (17, 18). Molecular heterogeneity has been reported for α-fetoprotein from human and other species (19). It is worthwhile to study the possible contribution of tyrosine sulfation, in addition to glycosylation (20), to the microheterogeneity of this protein.

Fibronectin secreted by HepG2 cells is of special interest because hepatocytes are suggested to be the main producer of plasma fibronectin (21), which plays important roles in various physiological processes (22). Secreted fibronectin isolated in the present study, although also of hepatic origin, displayed some structural differences from plasma fibronectin. HepG2 fibronectin was found to be composed of equal-sized subunits; plasma fibronectin has been characterized to be a heterodimer (23). Furthermore, the former migrated more slowly during NaDodSO$_4$ gel electrophoresis, indicating a higher molecular weight than that of plasma fibronectin. It is at present not known if these differences are due to changes (e.g., in protease processing) following oncogenic transformation of HepG2 cells, or if the two fibronectins are actually distinct translational products.

In conclusion, data from the present study suggest that, although not ubiquitous, protein sulfation was found to be rather frequent. Sulfation on both the polypeptide backbone (tyrosine residue) and the carbohydrate moiety was detected. Frequent occurrence of these covalent modifications seems to imply their importance in biological processes. Further studies are necessary to clarify their functional relevance.

This work was supported by National Institutes of Health Grant GM 13972-18 and American Cancer Society Grant BC-478 (F.L.), National Institutes of Health Grant AI 17080 (J.S.), and National Institutes of Health Grants HL 32678 and HL 09011 (S.Y. and C.M.R.).

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