UT Assay Procedure. The mixture of 5 μl of UT sample is allowed to incubate for a few minutes at 26°, then 5 μl of *E. coli* P_{IIA} (0.4 unit/ml) is added to initiate the uridylylation reaction. After a suitable incubation period, 70 μl of the deadenylylation reaction mixture preequilibrated at 26° is added to determine the amount of P_{IID} formed. After 5 min of incubation, 1 ml of the γ-glutamyltransferase mixture is added, and after another 4 min, the reaction is stopped by adding 1 ml of the stop mixture. The resulting solution is centrifuged, and its absorbance at 540 nm is recorded. The control tube is prepared with the UT sample by a similar procedure as the sample tube with the exception that the UT assay mixture is substituted by the UT assay blank mixture.

The procedures described above can be used to measure P_{IID} and UT activity in *S. typhimurium*,[43] *P. putida*,[44] and *K. aerogenes*.[45] This is possible because P_{IID} from those organisms stimulates the deadenylylation reaction catalyzed by *E. coli* AT_d and because *E. coli* P_{IIA} is uridylylated by UT activities from various organisms.

[11] Determination and Occurrence of Tyrosine *O*-Sulfate in Proteins

By WIELAND B. HUTTNER

The term "protein sulfation" describes the modification of proteins by covalent attachment of sulfate. One can distinguish between two principal types of protein sulfation. The first type is the covalent linkage of sulfate to amino acid residues of proteins, i.e., the primary modification of the polypeptide chain itself. Only one amino acid, tyrosine, has so far been shown to undergo this modification. The second type is the covalent linkage of sulfate to carbohydrate moieties of glycoproteins and proteoglycans. In structural terms this type of sulfation is a secondary modification of the polypeptide chain, the primary one being protein glycosylation. Both sulfated tyrosine and sulfated carbohydrate residues can apparently occur in the same protein.

The sulfate linked to tyrosine is present as an O^4-sulfate ester. Such a tyrosine O^4-sulfate residue was first identified in 1954 in bovine fibrinopeptide B.[1] In the 28 years following this discovery, the presence of

[1] F. R. Bettelheim, *J. Am. Chem. Soc.* **76,** 2838 (1954).

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tyrosine sulfate, initially believed to be restricted to fibrinogens and fibrins,[2] was detected in a few biological peptides, such as gastrin II,[3] phyllokinin,[4] caerulein,[5] cholecystokinin,[6] and Leu-enkephalin,[7] as well as in the small polypeptide hirudin.[8] Except for these peptides (and their precursors), however, the occurrence of tyrosine sulfate in proteins remained virtually unnoticed until recently. In fact, when proteins were found to be sulfated, this was, with a single exception,[9] generally taken to be indicative of the presence of sulfated carbohydrate residues.

In a more recent study,[10] the possible widespread occurrence of tyrosine sulfate in proteins was investigated. Tyrosine sulfate was detected in proteins from a wide variety of vertebrate tissues and cell cultures in many molecular weight ranges. Since this recognition of tyrosine sulfation as a widespread modification of proteins, at least 10 specific tyrosine-sulfated proteins have been newly identified and partially characterized. These are (1) the four major sulfated proteins of a rat pheochromocytoma cell line (PC12), designated as p113, p105, p86, and p84, which were also found to be phosphorylated on serine[11]; (2) the major soluble sulfated protein of the rat brain, designated as p120, which was found to be developmentally regulated[12]; (3) three prominent sulfated proteins of the chick retina, designated as p110, p102, and p93, which were found to move by fast axonal transport down the optic nerve[12], (4) an acidic secretory protein of the bovine anterior pituitary,[13] originally described by Rosa and Zanini[14]; (5) immunoglobulin G of some hybridoma cell lines[15]; and (6) vitellogenin of *Drosophila*.[15]

[2] F. R. Jevons, *Biochem. J.* **89,** 621 (1963).
[3] H. Gregory, P. M. Hardy, D. S. Jones, G. W. Kenner, and R. C. Sheppard, *Nature (London)* **204,** 931 (1964).
[4] A. Anastasi, G. Bertaccini, and V. Erspamer, *Br. J. Pharmacol. Chemother.* **27,** 479 (1966).
[5] A. Anastasi, V. Erspamer, and R. Endean, *Arch. Biochem. Biophys.* **125,** 57 (1968).
[6] V. Mutt and J. E. Jorpes, *Eur. J. Biochem.* **6,** 156 (1968).
[7] C. D. Unsworth, J. Hughes, and J. S. Morley, *Nature (London)* **295,** 519 (1982).
[8] T. E. Petersen, H. R. Roberts, L. Sottrup-Jensen, S. Magnusson, and D. Bagdy, *in* "Protides of Biological Fluids" (H. Peeters, ed.), Vol. 23, p. 145. Pergamon, Oxford, 1976.
[9] G. Scheele, D. Bartelt, and W. Bieger, *Gastroenterology* **80,** 461 (1981).
[10] W. B. Huttner, *Nature (London)* **299,** 273 (1982).
[11] R. W. H. Lee and W. B. Huttner, *J. Biol. Chem.* **258,** 11326 (1983); R. W. H. Lee, A. Hille, and W. B. Huttner, unpublished observations.
[12] S. B. Por and W. B. Huttner, manuscripts in preparation.
[13] P. Rosa, G. Fumagalli, A. Zanini, and W. B. Huttner, manuscript submitted for publication.
[14] P. Rosa and A. Zanini, *Mol. Cell. Endocrinol.* **24,** 181 (1981).
[15] P. A. Baeuerle and W. B. Huttner, manuscripts submitted for publication.

The role of tyrosine sulfation in the function of these proteins is the subject of current investigations. A possible common denominator may be the observation that all polypeptides so far known to contain tyrosine sulfate either are secretory proteins or show properties consistent with them being secretory proteins. It is therefore possible that tyrosine sulfation is involved in the processing, sorting, or functioning of some secretory proteins. In the case of the known proteins, tyrosine sulfation may occur during their passage through the Golgi complex; it appears to be only slowly reversible or even irreversible.[11]

It is, however, too early to draw general conclusions about the subcellular compartmentation and the degree of reversibility of tyrosine sulfation. The proteins mentioned above are only some of the tyrosine-sulfated proteins existing in the respective cell systems, and only a few cell systems have so far been studied. Clearly, many more tyrosine-sulfated proteins are yet to be discovered, and many more known proteins need to be tested for the presence of tyrosine sulfate. Hopefully, by learning more about tyrosine-sulfated proteins, we will achieve an understanding of the biological role(s) of this modification. In the following sections, some of the current procedures used to detect tyrosine-sulfated proteins and to study this modification are described.[16]

Chemical Synthesis and Properties of Tyrosine Sulfate

Synthesis. L-Tyrosine O^4-sulfate is synthesized according to the method of Reitz *et al.*[17] by the reaction of L-tyrosine with concentrated sulfuric acid at low temperature. After the reaction, the sulfuric acid is neutralized and precipitated by addition of barium hydroxide. The tyrosine sulfate is freed from unreacted tyrosine and Ba^{2+} by passage through a cation exchanger. The final product may contain variable amounts of tyrosine 3′-sulfonate, which can be distinguished from tyrosine sulfate by its slightly different electrophoretic mobility at pH 3.5, its distinct ultraviolet absorption spectrum, and its stability to acid at elevated temperature. The appearance of tyrosine 3′-sulfonate can be minimized (less than 1% of the tyrosine sulfate) by limiting the reaction time of tyrosine with sulfuric acid to 15 min and by keeping the reaction temperature low (between

[16] For recent overviews of sulfation, the reader is referred to two excellent books: G. J. Mulder, "Sulfation of Drugs and Related Compounds," CRC Press, Boca Raton, Florida, 1981; G. J. Mulder, J. Caldwell, G. M. J. Van Kempen, and R. J. Vonk, "Sulfate Metabolism and Sulfate Conjugation," Taylor & Francis, London, 1982.

[17] H. C. Reitz, R. E. Ferrel, H. Fraenkel-Conrat, and H. S. Olcott, *J. Am. Chem. Soc.* **68,** 1024 (1946).

−25 and −10°). Tyrosine O^4-[^{35}S]sulfate is synthesized by the same protocol, using [^{35}S]H_2SO_4.

Properties. One of the most remarkable properties of tyrosine sulfate is the lability of the ester bond in acid and its stability in alkali. More than 95% of the ester is hydrolyzed after 5 min in 1 *M* HCl at 100°, making it impossible to detect tyrosine sulfate after acid hydrolysis of proteins. The acid lability of the ester presumably explains why tyrosine sulfate is not observed when peptides known to contain this modified amino acid are sequenced by chemical methods. Fortunately, more than 90% of the ester remains after 24 hr in 0.2 *M* $Ba(OH)_2$ at 110°, making alkaline hydrolysis the method of choice to detect tyrosine sulfate in proteins. Tyrosine sulfate (Ba^{2+}, K^+, or Na^+ salt) is stable in neutral aqueous solution at 4° for weeks, and standard solutions can be kept frozen at −20° for at least a year. The ultraviolet absorption spectrum of tyrosine sulfate is different from that of tyrosine, showing a peak at 260.5 nm with a molar extinction coefficient of 283 (pH 7.0).[18]

Detection of Tyrosine Sulfate in Proteins

This chapter focuses on the detection of tyrosine-sulfated proteins and on the determination of tyrosine sulfate by methods that are based on labeling proteins with $^{35}SO_4$. This approach has several advantages over the chemical determination of tyrosine sulfate in unlabeled proteins. It can be much more easily used to search for tyrosine-sulfated proteins and is considerably more sensitive and therefore requires less protein material for analysis. An immunological approach to detect tyrosine-sulfated proteins has been developed in our laboratory.[18] Antisera were raised against a synthetic antigen containing a large number of tyrosine sulfate residues, and antibodies were purified from the antisera by affinity chromatography. These antibodies appeared to recognize tyrosine sulfate-containing proteins in "Western" blots and in solid-phase radioimmunoassay. The general usefulness of these antibodies to screen for, immunoprecipitate, or purify tyrosine-sulfated proteins is currently being tested.

The standard method to detect tyrosine sulfate in proteins involves four main steps described in Sections 1–4 below: (1) *in vivo* labeling of tissues *in situ* or of tissue explants, tissue slices, and cells in culture with inorganic [^{35}S]sulfate; (2) separation of proteins by polyacrylamide gel electrophoresis (PAGE); (3) elution from gels and hydrolysis of individual $^{35}SO_4$-labeled proteins; (4) separation of tyrosine [^{35}S]sulfate by thin-layer electrophoresis. Major modifications of and additions to the standard

[18] P. A. Baeuerle, Diploma Thesis, University of Konstanz, 1983.

steps are described in separate paragraphs at the end of each section. A general scheme of the various procedures is shown in Fig. 1.

1. Labeling with $^{35}SO_4$

When whole animals are labeled with $^{35}SO_4$, one should bear in mind that the isotope becomes distributed throughout the body and that its specific activity is reduced by the endogenous unlabeled sulfate. Thus, in order to achieve sufficient $^{35}SO_4$ incorporation into proteins of the tissue of interest, relatively large quantities of $^{35}SO_4$ of high specific activity (>900 Ci/mmol) should be used. For example, a single intraperitoneal injection of 20 mCi of $^{35}SO_4$ into a 100-g rat was sufficient for the detection of sulfated proteins in various tissues and in the plasma 18 hr after the injection by SDS–PAGE and fluorography of the gels for 30 hr.[10] More efficient labeling is found if the $^{35}SO_4$ is administered to the tissue of

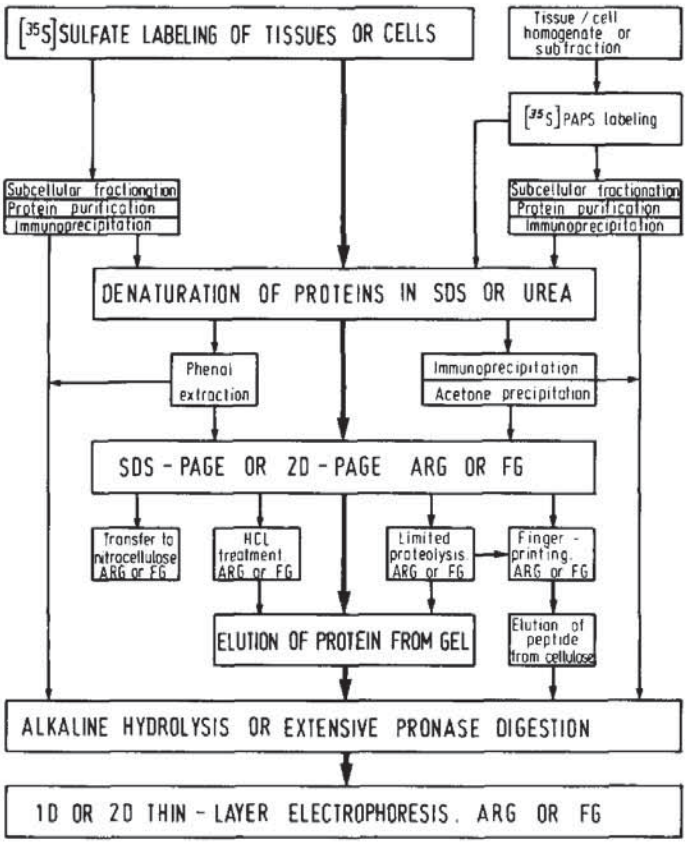

FIG. 1. Schematic outline of the sequence of procedures used to detect tyrosine sulfate in proteins. Thick arrows indicate the sequence of the standard method; thin arrows indicate modifications and additions. ARG, autoradiography; FG, fluorography.

interest directly. For example, sulfated proteins could easily be detected in the chick retina or the rat brain 1 hr after injection of 2 mCi of $^{35}SO_4$ into the chick eye or the third ventricle of the rat brain, respectively, by SDS-PAGE and fluorography of the gels for 15 hr.[12]

Mammalian tissues are not capable of reducing $^{35}SO_4$ for the synthesis of [^{35}S]methionine and [^{35}S]cysteine. Nevertheless, one encounters [^{35}S]methionine and [^{35}S]cysteine incorporation into proteins after labeling whole animals with $^{35}SO_4$. This is presumably due to the synthesis of [^{35}S]methionine and [^{35}S]cysteine by bacteria present in the gastrointestinal tract of the animals and can be prevented by using germfree animals. Although this phenomenon makes the interpretation of fluorograms of SDS–polyacrylamide gels less straightforward, it poses no problem for the identification of tyrosine [^{35}S]sulfate in protein hydrolysates, using thin-layer electrophoresis (see Section 4).

When tissue explants and cells are labeled in culture with $^{35}SO_4$, efficient $^{35}SO_4$ incorporation into proteins is achieved by adding carrier-free $^{35}SO_4$ to sulfate-free medium. Many culture systems use medium supplemented with some sort of serum, and the presence of low concentrations of unlabeled sulfate (up to about 10^{-4} *M*), as is the case when sulfate-free medium supplemented with undialyzed serum is used, still allows satisfactory radioactive sulfate incorporation into proteins. In this case, the reduction in the specific activity of $^{35}SO_4$ is presumably compensated to some extent by the increase in cellular sulfate uptake. The advantage of using the isotope at high specific activity should be balanced against the potential disadvantage that may result from the use of dialyzed serum and from starving cells of sulfate. It is therefore recommended to ascertain that the use of sulfate-free medium and (when serum is required for the culture) the use of dialyzed serum during $^{35}SO_4$ labeling does not reduce the viability of the cells under investigation. In particular, the capacity for protein synthesis should not be impaired, since protein synthesis appears to be required for suifate incorporation into some proteins.[11] In our experience, the use of carrier-free sulfate (about 0.5 mCi/ml final concentration) in sulfate-free medium supplemented with a reduced concentration (1–5%) of undialyzed serum has resulted in very efficient incorporation of radioactive sulfate into PC12 cell proteins after labeling periods of up to 18 hr.

Since incorporation of $^{35}SO_4$ into proteins may occur at both tyrosine residues and carbohydrate residues, it can be informative to perform $^{35}SO_4$ labeling in the absence and in the presence of inhibitors of N-glycosylation, e.g., tunicamycin. For example, in the slime mold *Dictyostelium discoideum,* protein sulfation was virtually abolished by tunicamycin, and analysis of sulfated proteins for the presence of tyrosine sulfate

gave essentially negative results.[19] In PC12 cells, the four proteins designated p113, p105, p86, and p84, known to contain most of the incorporated sulfate as tyrosine sulfate,[11] were labeled similarly with $^{35}SO_4$ in the absence and in the presence of tunicamycin.

Labeling with 3'-Phosphoadenosine 5'-Phospho[^{35}S]sulfate (PAPS). The study of tyrosine sulfation of proteins has been extended to cell-free systems.[11] Using the radiolabeled "activated sulfate" [^{35}S]PAPS as sulfate donor, transfer of $^{35}SO_4$ to tyrosine residues of endogenous protein acceptors, catalyzed by an endogenous tyrosylprotein sulfotransferase, can be observed in cell lysates and some subcellular fractions. At present, the labeling efficiency of proteins in cell lysates is less than that obtained in intact cells, for several reasons. (1) The specific activity of commercially available [^{35}S]PAPS is relatively low (~2 Ci/mmol), compared with that of inorganic [^{35}S]sulfate (>900 Ci/mmol). (2) The proportion of the protein of interest that is in the unsulfated form, and thus a substrate for labeling, may be small at any given time point including that of cell lysis, whereas in intact cells ongoing protein synthesis continuously supplies unsulfated substrate protein. There can be little doubt, however, that, as the components of cell-free tyrosine sulfation of proteins are elucidated, sulfation of defined proteins by tyrosylprotein sulfotransferase will become more efficient, and the results obtained will increasingly contribute to our understanding of the role of tyrosine sulfation.

2. *Separation of $^{35}SO_4$-Labeled Proteins*

After labeling of tissues or cell cultures with $^{35}SO_4$, reactions are terminated and proteins are solubilized for separation on polyacrylamide gels. For these purposes, the use of SDS together with boiling at neutral pH appears to be the most suitable method, since it fulfills all the following requirements.

1. Rapid inactivation of enzymes. Although at present little is known about the possible regulation of the sulfation of specific proteins by extracellular and intracellular signals, only the preservation of the *in vivo* state of protein sulfation by rapid enzyme inactivation may allow the observation of such regulatory phenomena.

2. Avoidance of low pH. The tyrosine sulfate ester is labile in acidic conditions at elevated temperatures. Although the ester bond may be stable in acidic conditions in the cold, it appears safer to avoid low pH

[19] J. Stadler, G. Gerisch, G. Bauer, C. Suchanek, and W. B. Huttner, *EMBO J.* **2,** 1137 (1983).

whenever possible. It is for this reason that we avoid the use of trichloroacetic acid for terminating sulfation reactions.

3. Complete solubilization of proteins. Proteins solubilized in SDS can be subjected to phenol extraction, immunoprecipitation, SDS–PAGE and, after acetone precipitation, two-dimensional PAGE (see Fig. 1).

An SDS-containing, neutral solution that we have found to be suitable for most purposes is the sample buffer according to Laemmli,[20] referred to as "stop solution." Cells attached to culture dishes and cell pellets are directly dissolved in stop solution [3% (w/v) SDS, 10% (w/v) glycerol, 3.3% (v/v) 2-mercaptoethanol, a trace of bromophenol blue, and 62.5 m*M* Tris-HCl, pH 6.8], whereas cells in suspension are mixed with 0.5 volume of three times concentrated stop solution, followed in both cases by immediate boiling of the samples for 3–5 min. Tissues are rapidly frozen in liquid nitrogen, crushed into a fine powder under liquid nitrogen, and then dissolved in stop solution followed by boiling.

After $^{35}SO_4$ labeling, tissues and cells may be subjected to subcellular fractionation, and the subcellular fractions of interest can then be dissolved in stop solution. It should be borne in mind, however, that rapid regulatory phenomena in the sulfation of proteins, should they exist, might be lost during the fractionation process.

In an attempt specifically to remove sulfated carbohydrate residues from proteins after $^{35}SO_4$ labeling and at the same time preserve tyrosine sulfate residues, we have subjected PC12 cell proteins, known to contain tyrosine sulfate,[11] to treatment with anhydrous hydrogen fluoride according to Mort and Lamport.[21] In our experience, however, all the sulfate was removed from these proteins by this treatment. We have not used enzymatic deglycosylation to distinguish between the presence of sulfated carbohydrate residues and tyrosine sulfate residues, but this may be useful in some cases.

If specific proteins are to be subjected to immunoprecipitation prior to electrophoresis, cells and tissues can be dissolved in a neutral buffer without 2-mercaptoethanol containing 1–3% (w/v) SDS followed by boiling. The SDS is then diluted by addition of the nonionic detergent Nonidet P-40, and immunoprecipitation is performed according to standard procedures.[22] Alternatively, cells can be dissolved in RIPA buffer and subjected to immunoprecipitation as described.[23] Immunoprecipitates can be dis-

[20] U. K. Laemmli, *Nature (London)* **227,** 680 (1970).
[21] A. J. Mort and D. T. A. Lamport, *Anal. Biochem.* **82,** 289 (1977).
[22] S. E. Goelz, E. J. Nestler, B. Chehrazi, and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2130 (1981).
[23] B. M. Sefton, K. Beemon, and T. Hunter, *J. Virol.* **28,** 957 (1978).

solved in stop solution (for SDS–PAGE) or in O'Farrell lysis buffer[24] (for two-dimensional PAGE).

Cells and tissues dissolved in stop solution can be subjected to any of the following protocols.

1. A phenol extraction protocol as described in Procedure I, designed to separate the proteins from sulfated glycosaminoglycans, followed by PAGE (see below). This procedure is similar to that introduced by Hunter and Sefton[25] for the study of tyrosine phosphorylation of proteins, and used in that case to separate proteins from nucleic acids and phospholipids. The effect of phenol extraction is illustrated in Fig. 2.
2. SDS–PAGE according to Laemmli.[20]
3. Two-dimensional PAGE using either isoelectric focusing or nonequilibrium pH gradient electrophoresis in the first dimension, as described by O'Farrell.[24] For two-dimensional PAGE, samples in stop solution are mixed with 5 volumes of acetone, kept at −20° until precipitation occurs, and centrifuged. The pellets are washed in 80% (v/v) acetone, dried, and dissolved in O'Farrell lysis buffer containing 5% (w/v) Nonidet P-40. Alternatively, cells attached to culture dishes or cell pellets can also be directly dissolved into lysis buffer. In our experience, both types of sample preparation give rise to similar separations upon two-dimensional PAGE.

Procedure I. Phenol Extraction of Sulfated Proteins

1. Dissolve sample, e.g., $^{35}SO_4$-labeled cells in culture, in stop solution (0.5–5 mg of protein per milliliter of stop solution). Boil immediately for 3–5 min.
2. Unless otherwise indicated, the following steps are performed at room temperature. Prepare phenol solution: dissolve phenol in an equal amount (w/v) of HEN buffer (50 m*M* HEPES-NaOH, pH 7.4; 5 m*M* EDTA; 100 m*M* NaCl), mix vigorously for 5 min, let stand or centrifuge until phases are separated. The lower phase is HEN buffer-saturated phenol (referred to as phenol solution), the upper phase is phenol-saturated HEN buffer (referred to as HEN solution).
3. Mix the sample with an equal volume of phenol solution, vortex vigorously for at least 30 sec, centrifuge for 10 min at 15,000 rpm in a Sorvall SS34 rotor.

[24] P. H. O'Farrell, *J. Biol. Chem.* **250,** 4007 (1975); P. Z. O'Farrell, H. M. Goodman, and P. H. O'Farrell, *Cell* **12,** 1133 (1977).
[25] T. Hunter and B. M. Sefton, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1311 (1980).

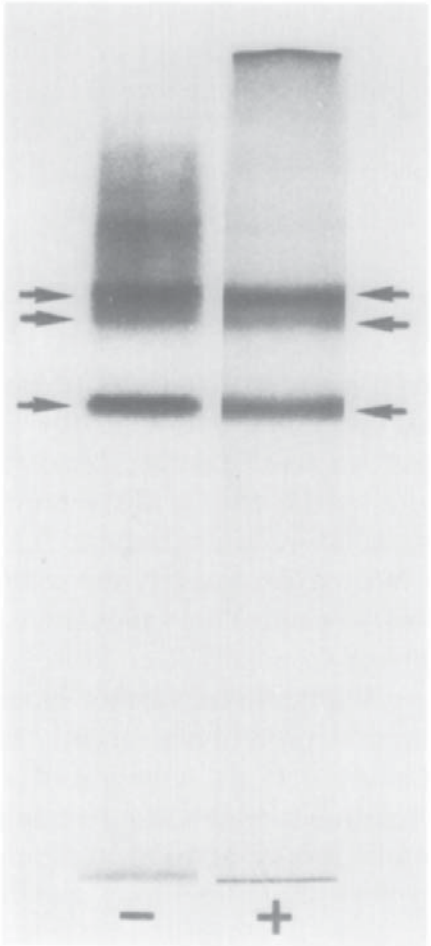

FIG. 2. Autoradiograms showing the effect of the phenol extraction protocol described in Procedure I. PC12 cells were labeled with $^{35}SO_4$ and subjected to SDS–PAGE, without (−) or with (+) prior phenol extraction. Arrows indicate major tyrosine-sulfated proteins.[11] Some of the proteins can be better seen after phenol extraction.

4. Collect the aqueous (upper) phase. If flocculent material at the interface is present, avoid disturbing it. Keep phenol (lower) phase plus interface.

5. Mix the aqueous phase with an equal volume of phenol solution; vortex and centrifuge as before. Discard the aqueous phase; keep the phenol phase plus interface.

6. Pool the phenol phases plus interfaces from first and second extraction; reextract 1–3 times with equal volume of HEN solution. Discard aqueous phases each time. The efficiency of extraction can be monitored by spotting aliquots of the aqueous phases on filter paper and observing the decline in radioactivity with a portable β-radiation monitor or by liquid scintillation counting.

7. Add five volumes of cold (−20°) ethanol to final phenol phase plus interface. If necessary, transfer to larger centrifuge tube. Mix well and keep at −20° until precipitation has occurred (2 hr or longer). Centrifuge for 10 min at 10,000 *g* in a Sorvall SS34 rotor. Discard the supernatant. Wash the precipitate once with chloroform–methanol (2 : 1) and collect it by centrifugation as above. Allow the precipitate to dry.

8. Dissolve the precipitate in stop solution (for SDS–PAGE), in lysis buffer (for two-dimensional PAGE), or in 0.2 *M* $Ba(OH)_2$ (for alkaline hydrolysis and determination of tyrosine sulfate, see Procedure III).

After electrophoresis, gels are fixed and (if desired) stained and destained by conventional procedures, using acetic acid rather than trichloroacetic acid. Fixed gels can also be subjected to an acid treatment that is described in a subsection at the end of this section. For the detection of $^{35}SO_4$-labeled proteins, gels are prepared for fluorography, dried, and fluorographed at −70°. We use exclusively the sodium salicylate method[26] for fluorography, primarily because the salicylate is water-soluble and can therefore easily be removed from gels after fluorography. This renders the proteins present in the gel suitable for further biochemical analysis, e.g., peptide mapping by limited proteolysis, tryptic fingerprinting, tyrosine sulfate analysis, (see below). If the labeling of proteins is sufficiently intense, the salicylate treatment of gels can be omitted, and the dried gels are subjected to autoradiography at room temperature. The X-ray film used in our laboratory for autoradiography and fluorography is Kodak XAR-5.

An alternative, rapid procedure to obtain an autoradiogram after SDS–PAGE or two-dimensional PAGE is to transfer the proteins from the gel onto nitrocellulose filter paper using the "Western" blotting technique. After the transfer, the nitrocellulose filter paper is either air-dried and autoradiographed or is dipped in 20% PPO in toluene, dried, and fluorographed at −70°. This procedure has two advantages.

1. The time needed for autoradiography after transfer to nitrocellulose filter paper is shorter than with dried polyacrylamide gels.

2. The sulfated material that is often found as a diffuse smear in the high molecular weight regions of SDS–polyacrylamide gels (presumably sulfated proteoglycans or glycosaminoglycans) does not appear to transfer very well from the gel to the nitrocellulose filter paper. Thus, autoradiograms obtained after transfer of $^{35}SO_4$-labeled proteins to nitrocellulose filter paper tend to be "cleaner" than those of the corresponding gels

[26] J. P. Chamberlain, *Anal. Biochem.* **98,** 132 (1979).

and can be compared to autoradiograms obtained after SDS–PAGE of phenol-extracted samples (Fig. 2).

$^{35}SO_4$-labeled proteins separated in polyacrylamide gels can be used for hydrolysis (see Section 3 below) and tyrosine sulfate analysis (Section 4). If desired (see Fig. 1), individual sulfated protein bands can first be subjected to limited proteolysis in SDS and peptide mapping in SDS polyacrylamide gels,[27] or to extensive proteolysis and two-dimensional fingerprinting by thin-layer electrophoresis/chromatography. In these cases, the resulting peptide fragments can be used for hydrolysis and tyrosine sulfate analysis.

HCl Treatment of Proteins in Polyacrylamide Gels. The details of the method are described in Procedure II, below. The rationale of this treatment is to screen, among the variety of sulfated proteins present in a sample, for those likely to contain tyrosine sulfate. Since the tyrosine sulfate ester is remarkably acid-labile and appears to be hydrolyzed faster than most carbohydrate sulfate esters, a short acid treatment will lead to a preferential loss of sulfate from tyrosine residues. When such an acid treatment is performed on $^{35}SO_4$-labeled proteins fixed in polyacrylamide gels, the resulting autoradiographic pattern may show some labeled bands that disappear quite specifically after the acid treatment (i.e., their reduction is greater than the small overall reduction in labeled bands). These specifically acid-sensitive bands may contain a large proportion of their sulfate label as tyrosine sulfate. In the examples illustrated in Fig. 3, there appears to be a good correlation between the acid sensitivity of bands and their tyrosine sulfate content.

It may, however, be too early to assume generally that a correlation between these two parameters will be found in every case because one cannot rule out false-positive and false-negative results. Possible explanations for false-positive acid sensitivity of bands include the following. (1) Sulfated residues other than tyrosine sulfate, e.g., certain carbohydrate sulfate esters, may exist that are as acid-sensitive as tyrosine sulfate ester. (2) The acid treatment may lead to hydrolysis of some peptide bonds. This may result in the formation of peptide fragments small enough to remain no longer fixed in the gel but to diffuse out. If sulfated residues other than tyrosine sulfate, e.g., sulfated carbohydrates, were located in such peptide fragments, an acid treatment-induced loss of $^{35}SO_4$ label would be observed without the actual hydrolysis of a tyrosine sulfate ester. False-negative acid sensitivity, i.e., the apparent lack of acid sensi-

[27] D. W. Cleveland, S. G. Fischer, M. W. Kirschner, and U. K. Laemmli, *J. Biol. Chem.* **252,** 1102 (1977).

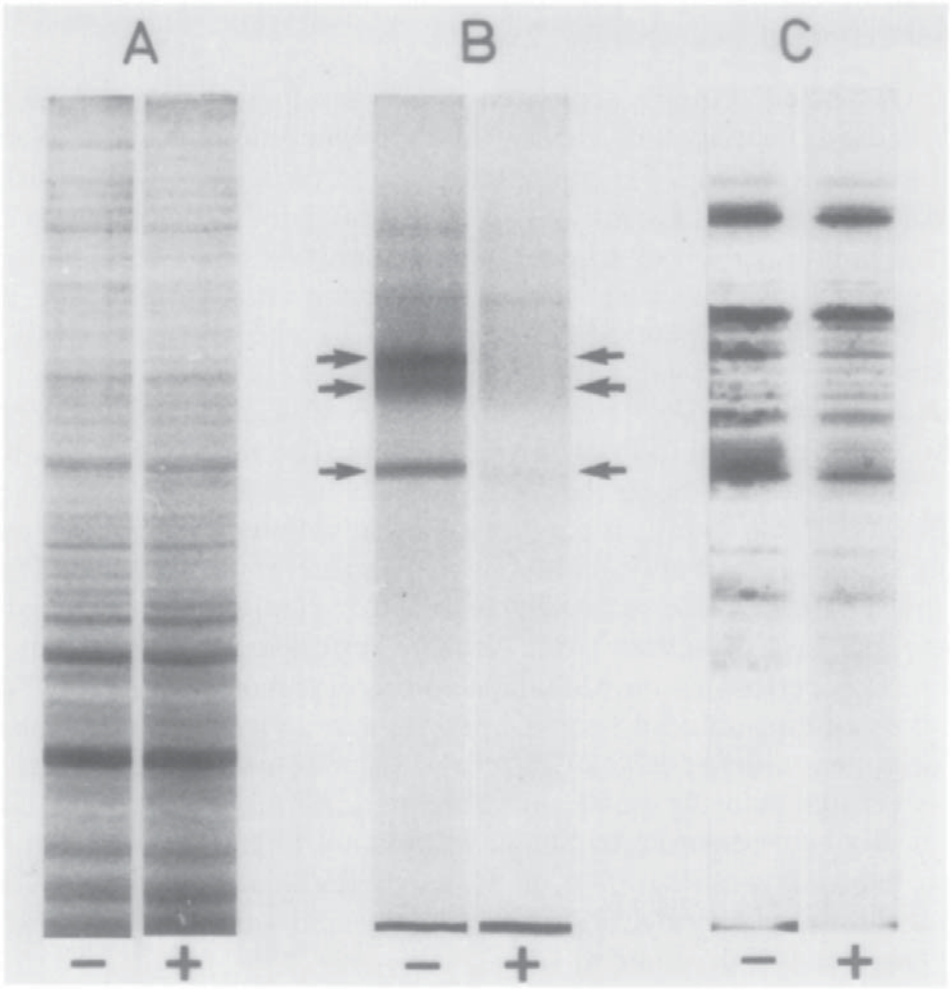

FIG. 3. Autoradiograms showing the effect of the HCl treatment described in Procedure II on proteins separated by SDS–PAGE: (A) proteins of rat pheochromocytoma cells (PC12), labeled with [^{35}S]methionine; (B) same, labeled with [^{35}S]sulfate; (C) proteins of *Dictyostelium discoideum*, labeled with [^{35}S]sulfate. Compared with the control (−), the HCl treatment (+) does not lead to a loss of protein from the gel (A). The HCl treatment markedly reduces the $^{35}SO_4$ label of PC12 cell proteins known to contain tyrosine sulfate[11] (arrows) (B), but only slightly the $^{35}SO_4$ label of *D. discoideum* proteins known to contain sulfate on carbohydrate residues[19] (C).

tivity of bands despite the presence of tyrosine sulfate, may also be observed. For example, if a glycoprotein contained 10 sulfated carbohydrate residues and 1 tyrosine sulfate residue, the acid treatment-induced specific loss of $^{35}SO_4$ label of about 10% would not readily be noticed upon comparative autoradiography. Despite these considerations, the acid treatment of gels is a useful and simple tool in searching for tyrosine-sulfated proteins, particularly when this method is combined with the

procedure to determine tyrosine sulfate in individual proteins obtained from polyacrylamide gels (Sections 3 and 4).

Procedure II. Acid Treatment of Sulfated Proteins after Separation in Polyacrylamide Gels

1. This method can be applied to one- or two-dimensional wet polyacrylamide gels after electrophoresis, fixation, and (optional) Coomassie Blue staining, as well as to dried gels after autoradiography or fluorography using sodium salicylate. Wet gels are fixed after electrophoresis in 10% acetic acid–50% methanol for at least 1 hr. The same solution containing Coomassie Blue can be used simultaneously to fix and to stain the gel, if staining is required to cut a selected portion from the gel for acid treatment. Dried gels or portions of dried gels, after autoradiography or fluorography using sodium salicylate, are reswollen in 10% acetic acid–30% methanol for at least 1 hr. If the dried gel contained sodium salicylate, the solution should be changed at least once.

2. Equilibrate the gel in cold 1 *M* HCl for 30 min with constant shaking on ice. Use 10 volumes of HCl per original gel volume. While the gel is equilibrating, heat another 10 volumes of 1 *M* HCl in a separate, covered container that is placed in a boiling water bath. Make sure that this HCl solution has reached the final temperature (~90°) at the end of the equilibrating period.

3. Transfer the gel from the cold HCl into the container with the hot (~90°) HCl. Gently shake the container with the gel in the boiling water bath for 5 min. After 5 min, remove the container with the gel from the water bath and carefully pour the hot HCl and the gel into a large (10 liter) tray with ice water to stop the hydrolysis.

4. After a few minutes in ice water, place gel either into 10% acetic acid–50% methanol (if staining–destaining is not required), or into the same solution containing Coomassie Blue followed by destaining in 10% acetic acid–10% isopropanol (if staining–destaining is required), or directly into destaining solution (if gel had been stained prior to acid treatment). A minimum of 3 hr with constant shaking should be allowed for diffusion of hydrolyzed $^{35}SO_4$ from the gel.

5. Rinse the gel in water for 15 min and dry (if fluorography is not desired), or rinse in water, then shake in 1 *M* sodium salicylate for 30 min, and then dry (if fluorography is desired).

3. *Hydrolysis of $^{35}SO_4$-Labeled Proteins Obtained from Polyacrylamide Gels*

The method for the hydrolysis of proteins from polyacrylamide gels to liberate tyrosine sulfate is described in detail in Procedure III, below. Individual proteins contained in polyacrylamide gels are eluted by pro-

RECOVERY OF TYROSINE SULFATE AND SULFATED CARBOHYDRATES AFTER HYDROLYSIS WITH BARIUM HYDROXIDE

$^{35}SO_4$-labeled sample	Eluate (Procedure III, step 3)	Neutralized supernatant (Procedure III, step 6)
Tyrosine sulfate[a]	611 cpm 100%	576 cpm 94%
Contact site A glycoprotein of *Dictyostelium discoideum*[b]	1526 cpm 100%	3 cpm <1%
Hemagglutinin of influenza virus[c]	553 cpm 100%	4 cpm <1%

[a] Lee and Huttner.[11]
[b] Stadler *et al.*[19]
[c] Nakemura and Compans, and Huttner and Matlin.[28]

teolytic digestion. Since the proteolytic digestion serves only to facilitate the elution of the protein, the extent of digestion and the sites of cleavage in the protein are irrelevant, as long as the labeled peptide fragments are sufficiently small to diffuse out from the gel. We have used both trypsin and Pronase, with qualitatively similar results. The efficiency of elution may be slightly greater with Pronase. We routinely preincubate the Pronase solution to inactivate possible contaminating enzymes (e.g., a hypothetical sulfatase) by proteolytic digestion and have not observed any loss of tyrosine sulfate during Pronase digestion. The eluate obtained after proteolytic digestion is subjected to exhaustive alkaline hydrolysis. As mentioned above, the tyrosine sulfate ester is acid-labile and alkali-stable, making alkaline hydrolysis the method of choice to complete the liberation of tyrosine sulfate from the polypeptide chain. For alkaline hydrolysis barium hydroxide is used, followed by neutralization with sulfuric acid. The advantage of using barium hydroxide is that inorganic radioactive sulfate, generated by hydrolysis of alkali-labile sulfated carbohydrate residues of glycoproteins and proteoglycans, is precipitated as barium sulfate, whereas tyrosine sulfate remains in solution. This is exemplified by the observation (see the table) that from the 80-kilodalton contact site A glycoprotein of *Dictyostelium discoideum* and the hemagglutinin of influenza virus, proteins known to contain sulfate on carbohydrate residues,[19,28] virtually none of the radioactive $^{35}SO_4$ originally present is re-

[28] K. Nakamura and R. W. Compans, *Virology* **86,** 432 (1978); W. B. Huttner and K. Matlin, unpublished observations.

covered in the supernatant obtained after neutralization of the alkaline hydrolysate. In contrast, approximately 90% of tyrosine [^{35}S]sulfate standard is recovered in the neutralized supernatant. Thus most, if not all, of the ^{35}S radioactivity that is recovered in the neutralized supernatant after hydrolysis of $^{35}SO_4$-labeled proteins with barium hydroxide is in the form of tyrosine sulfate. The exact proportion of ^{35}S in the neutralized supernatant that is present as tyrosine sulfate is determined after thin-layer electrophoresis, which is described in Section 4, below.

Procedure III. Liberation of Tyrosine Sulfate from Proteins Separated in Polyacrylamide Gels

1. The starting material is protein contained in one- or two-dimensional polyacrylamide gels. The gels may be wet or dried, stained or unstained, may contain the proteins noncovalently fixed (use acetic acid) or unfixed, and may or may not have been treated with sodium salicylate.

2. Locate the protein of interest according to the Coomassie Blue staining or the autoradiogram. Excise the portion of the gel that contains the desired protein and equilibrate (wet gel piece) or reswell (dried gel piece) in 10–20 ml of 10% acetic acid–30% methanol. (This and the following volume specifications are valid for gel pieces that contain up to 200 μl of fluid in the wet state. If larger gel pieces are used, increase volumes in steps 2 and 3 accordingly.) Remove filter paper, if present. Shake for 3 hr with a change of solution every hour. This serves to remove sodium salicylate, SDS, running buffer, excess Coomassie Blue, etc., while keeping the protein fixed in the gel. Wash gel piece in H_2O for 30 min to remove most of the acetic acid and methanol, and lyophilize the washed gel piece. If the gel piece is excised from a wet gel that has been stained and destained, it can be put directly in H_2O and then dried. If the gel piece is excised from a dried gel that has been stained, destained, and does not contain salicylate, it can be taken directly to step 3.

3. Make fresh Pronase solution: 50 m*M* NH_4HCO_3, pH 8, 50 μg/ml Pronase (Boehringer), and a trace of phenol red. Preincubate Pronase solution for 60 min at 37° in a capped tube. Reswell the dried gel piece in 1 ml of preincubated Pronase solution and incubate in capped tube with shaking for 12–24 hr at 37°. (The phenol red indicator can be used to monitor that no significant amount of acid remained in the gel piece.) Collect the eluate, thereby measuring its volume, into disposable tubes holding 3–4 ml. (These disposable tubes must tolerate the subsequent steps, i.e., centrifugation at 10,000 *g* and pH 14 at 110°; they must also be suitable for gastight sealing. We use uncapped 3-ml glass centrifuge tubes.) *Add another 1 ml of fresh, preincubated Pronase solution to gel piece and incubate for another 6–12 hr as before. Collect the second*

eluate and pool with first eluate. Of the pooled eluate, take a 5% aliquot and determine the radioactivity by liquid scintillation counting (use Pronase solution as blank). Lyophilize pooled eluate.

4. Make fresh 0.2 *M* $Ba(OH)_2$ (use degassed H_2O to reduce formation of $BaCO_3$). Usually, the barium hydroxide does not dissolve completely. While it is being stirred, take 1 ml of the barium hydroxide solution and add it to residue of lyophilized eluate. Seal the tube under N_2 so that it is gastight (for uncapped glass tubes: freeze sample, evacuate, seal by melting in flame). Place in an oven at 110° for 20–24 hr.

5. Cool the sample to 4° and break the seal. Centrifuge for 10 min at 10,000 *g*. Collect the supernatant and transfer it to another centrifuge tube. *Add 0.4 ml of H_2O to pellet, vortex, centrifuge as above, pool the second supernatant with first one*. Discard the tube with the pellet.

6. Neutralize the pooled supernatant with sulfuric acid, using the phenol red in the sample as indicator. Carefully add 1 *M* sulfuric acid with frequent vortexing until the phenol red turns transiently yellow at the site of sulfuric acid addition. Then continue the neutralization with 0.1 *M* sulfuric acid until the sample is homogeneously red-orange (pH ~7). If too much sulfuric acid has been added accidentally (yellow color), back-titrate with barium hydroxide. Centrifuge the sample for 10 min at 10,000 *g*. Collect the supernatant, thereby measuring its volume, and transfer to a 2-ml Eppendorf tube. *Add 0.4 ml of H_2O to pellet, vortex, centrifuge as above, pool second supernatant with first one*. Discard the tube with the pellet. The pooled supernatant is referred to as neutralized supernatant. Take a 5% aliquot of neutralized supernatant and determine the radioactivity by liquid scintillation counting. Lyophilize neutralized supernatant.

7. The residue of the neutralized supernatant is used for the identification of tyrosine sulfate by thin-layer electrophoresis as described in Procedure IV and Fig. 4.

8. In the above procedure, the steps given in *italics* can be omitted if only qualitative results are required.

Since of the $^{35}SO_4$ originally present in proteins mainly tyrosine [^{35}S]sulfate appears to be recovered in the neutralized supernatant, the proportion of ^{35}S in the neutralized supernatant to that in the eluate can be taken as a rough estimate of what proportion of the total sulfate in a protein is tyrosine sulfate. If proteins contain ^{35}S not only as sulfate, but also as methionine and cysteine (see Section 1, above), such an estimation is not valid. If it can be established for a given protein that most or all of the incorporated $^{35}SO_4$ is present as tyrosine sulfate, it is obvious that hydrolysis and tyrosine sulfate analysis need not be done routinely.

The alkaline hydrolysis of $^{35}SO_4$-labeled protein with barium hydroxide is not restricted to proteins contained in polyacrylamide gels, but is

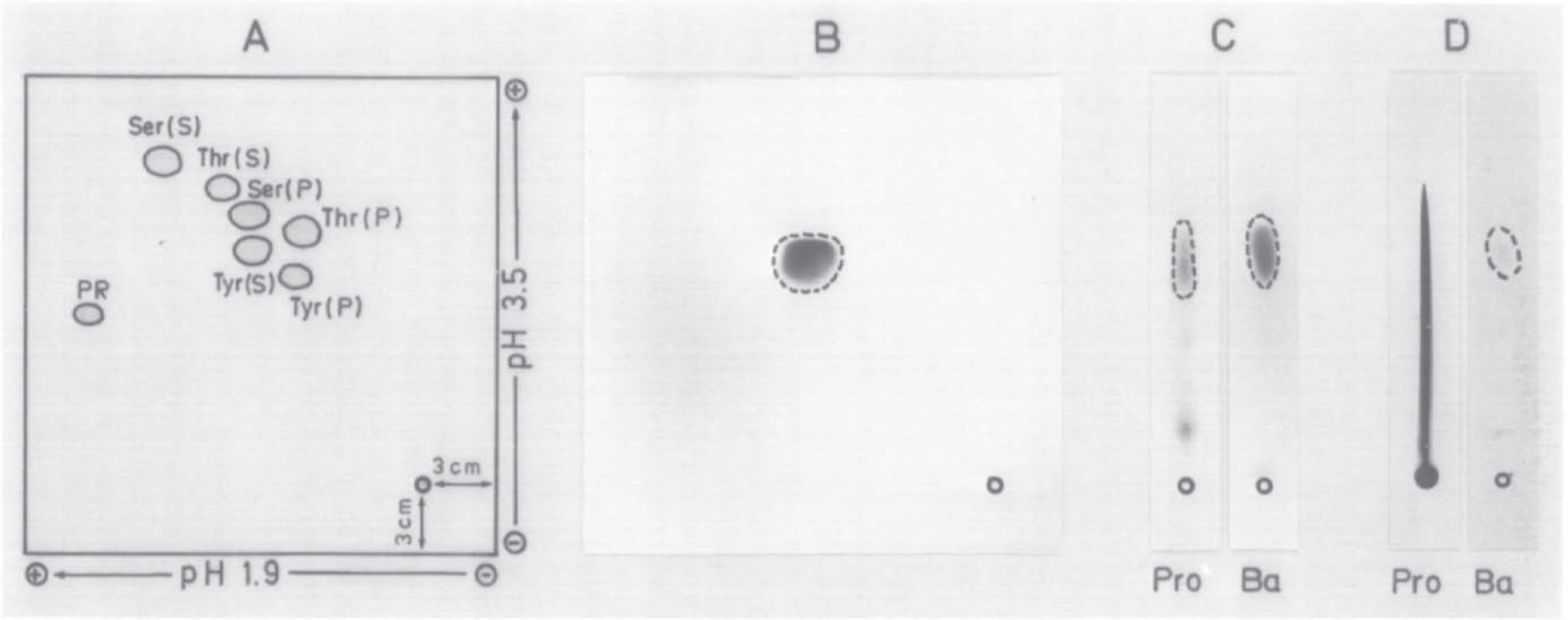

FIG. 4. Thin-layer electrophoresis of tyrosine sulfate. (A) Schematic diagram of the two-dimensional separation, giving the positions of sulfated and phosphorylated hydroxyamino acids. PR, Phenol red. (B) Autoradiogram of a two-dimensional separation of an alkaline hydrolysate (see Procedure III) of sulfated PC12 cell proteins. (C and D) One-dimensional separation and autoradiography comparing alkaline hydrolysates (Ba) with extensive Pronase digests (Pro). (C) A protein that contains sulfate predominantly as tyrosine sulfate[13]; (D) sulfated proteoglycan. Small circles in A, B, C, and D indicate the origin; the dotted lines in B, C, and D indicate the position of tyrosine sulfate.

applicable to any type of protein sample (immunoprecipitates, purified proteins, etc.). In the case of whole tissue or cells, it is advisable to carry the sample through the phenol extraction protocol (see Procedure I) to remove most of the nonprotein material prior to hydrolysis and thus to prevent overloading of the cellulose thin-layer sheet (see Section 4, below). Other protein samples can be prepared for hydrolysis by precipitation with acetone (85% final concentration).

Extensive Pronase Digestion of Proteins. The presence of tyrosine sulfate in proteins can also be detected after extensive proteolytic digestion, avoiding the strongly alkaline conditions described in Procedure III. The lyophilized pooled eluate of the desired $^{35}SO_4$-labeled protein, as obtained after step 3 of Procedure III, is dissolved in 50 μl of preincubated "high Pronase solution" (1 mg of Pronase per milliliter in 50 m*M* ammonium bicarbonate) and incubated for 12–24 hr at 37°. Thereafter, five volumes of acetone are added to precipitate excess Pronase, the precipitate is removed by centrifugation, the supernatant is collected, the acetone is evaporated, and the remainder is lyophilized. The residue is dissolved in pH 1.9 or pH 3.5 electrophoresis buffer and subjected to two-dimensional or one-dimensional thin-layer electrophoresis (described in step 4, Procedure IV), respectively, followed by autoradiography. Because of its mild nature, an extensive Pronase digestion is useful if the overall spectrum of sulfated residues of a protein is to be studied. This is further discussed in Section 4.

In some cases, a comparison of the result obtained by extensive Pronase digestion with that of the standard alkaline hydrolysis (see Procedure III) can be informative. For example, if a large proportion of the radioactive sulfate incorporated into a protein is lost during the hydrolysis with barium hydroxide and neutralization with sulfuric acid, this apparently indicates the presence of sulfated carbohydrate residues in excess over sulfated tyrosine residues. Such a loss would, however, be observed also if tyrosine sulfate residues of proteins, in contrast to tyrosine sulfate standard, were for some reason unstable during alkaline hydrolysis. In this case, the extensive Pronase digestion should give greater recovery of tyrosine sulfate than the alkaline hydrolysis. (We have not observed such a case so far, but cannot rule out its existence.)

4. Thin-Layer Electrophoresis of Tyrosine Sulfate

Thin-layer electrophoresis of tyrosine sulfate is performed under similar conditions as that of tyrosine phosphate[25] and is described in Procedure IV and Fig. 4. As discussed in Section 3, the hydrolysis and neutralization conditions used to liberate tyrosine sulfate from proteins render tyrosine sulfate the major, if not the only, ^{35}S-labeled substance in the

sample. One-dimensional thin-layer electrophoresis therefore suffices in most instances to demonstrate the presence of tyrosine sulfate. In fact, once it has been established for the protein of interest that most or all of the ^{35}S radioactivity in the neutralized supernatant is in the form of tyrosine sulfate, the electrophoretic separation may be omitted for routine analysis. If total cell protein rather than individual proteins have been used for alkaline hydrolysis, ^{35}S-labeled material other than tyrosine sulfate can be present in the neutralized supernatant, in which case the two-dimensional thin-layer electrophoresis can be used to identify unequivocally tyrosine sulfate.

Procedure IV. Thin-Layer Electrophoresis of Tyrosine Sulfate

A. One-Dimensional Electrophoresis

1. Dissolve the sample (the residue after lyophilization of the neutralized supernatant, see Procedure III) in 20 μl of pH 3.5 electrophoresis buffer (5% acetic acid–0.5% pyridine). If the residue is small and dissolves readily, the sample will usually run well in the thin-layer electrophoresis without prior acetone precipitation; proceed to step 3. If the residue appears to be too big to be loaded on a thin-layer sheet or does not dissolve readily, do an acetone precipitation as described in step 2.

2. Add 100 μl of acetone to a 20-μl sample. Sediment the precipitate by centrifugation (Microfuge, 5 min), collect the supernatant, and evaporate the acetone.

3. Spot the sample on 20 × 20 cm plastic-backed 100-μm cellulose thin-layer sheets (e.g., Schleicher & Schüll F 1440), 3 cm from the cathodic edge of the sheet (see Fig. 4); let dry. We have run up to 10 samples in parallel per sheet. Each sample spot should contain 3 μg of unlabeled tyrosine sulfate as marker, mixed to the sample prior to spotting, and a trace of phenol red (usually already present from the hydrolysis).

4. Wet the cellulose sheet with pH 3.5 electrophoresis buffer. This can be done either by spraying (if the spot is small) or by using a "mask" of Whatman 3 MM filter paper (if the spot is to be concentrated). In the latter case, take a piece of the filter paper that is slightly larger than the cellulose sheet, cut a circular hole, slightly larger than the sample spot, at the position corresponding to that of the sample on the cellulose sheet, wet the filter paper with electrophoresis buffer and place it on the cellulose sheet such that the sample spot is not touched by the filter paper. Allow the electrophoresis buffer to concentrically migrate toward the center of the spot, thereby concentrating the sample (watch the phenol red present in the sample). The cellulose sheet should be homogeneously wet, appearing gray without "shiny" areas that are indicative of excess buffer. Place wet cellulose sheet in electrophoresis apparatus.

5. Perform electrophoresis at pH 3.5 until the phenol red marker has migrated 7–8 cm, i.e., just past the middle of the cellulose sheet. In our electrophoresis system, this takes about 40,000 V × minutes, e.g., 80 min at 500 V. Tyrosine sulfate migrates approximately 1.5 times as fast as the phenol red under these conditions and is then usually found 5–7 cm from the anodic edge of the cellulose sheet.

6. Dry the cellulose sheet. The unlabeled tyrosine sulfate marker is then detected by spraying the cellulose sheet with 1% ninhydrin in acetone and developing the color in an oven at 100° for a few minutes. (If the radioactive tyrosine sulfate of the sample is to be preserved for further biochemical analysis, omit the ninhydrin staining.) Perform autoradiography or fluorography of the cellulose sheet to detect the radioactive tyrosine sulfate. For fluorography, method 1 of Bonner and Stedman[29] is used.

B. Two-Dimensional Electrophoresis

1. As in A1, except that pH 1.9 electrophoresis buffer (7.8% acetic acid–2.2% formic acid) is used.

2. As in A2, if necessary.

3. Spot on cellulose sheet 3 cm from the "bottom" edge (the cathodic edge in the second dimension) and 3 cm from the "right" edge (the cathodic edge in the first dimension (see Fig. 4A). Otherwise as in A3.

4. As in A4, using pH 1.9 electrophoresis buffer and a mask of filter paper to wet the sample.

5. Perform electrophoresis at pH 1.9 until the phenol red marker has reached a position approximately 2 cm from the anodic "left" edge of the cellulose sheet. In our electrophoresis system, this takes about 82,500–90,000 V × minutes, e.g., 110–120 min at 750 V. Tyrosine sulfate migrates approximately 0.5 times as fast as the phenol red under these conditions and is then usually found in the middle between the anodic and the cathodic edge of the cellulose sheet. Dry the cellulose sheet.

6. Wet the cellulose sheet with pH 3.5 buffer by spraying. Then perform electrophoresis at pH 3.5 as in A5 (the cathodic and anodic edges being "bottom" and "top" of the cellulose sheet, respectively).

7. Detect tyrosine sulfate as in A6.

The extensive Pronase digestion protocol described in Section 3 usually liberates at least 50%, but rarely more than 80%, of the tyrosine sulfate residues from proteins, the rest remaining in the form of small acidic peptides that are seen as discrete spots between the tyrosine sulfate spot and the origin after one-dimensional thin-layer electrophoresis and autoradiography (see Fig. 4C). In contrast to the alkaline hydrolysis, the extensive Pronase digestion does not destroy sulfated carbohydrate resi-

[29] W. M. Bonner and J. D. Stedman, *Anal. Biochem.* **89**, 247 (1978).

dues of glycoproteins and proteoglycans. When extensive Pronase digests of $^{35}SO_4$-labeled glycoproteins and proteoglycans are analyzed by one-dimensional thin-layer electrophoresis and autoradiography, one sometimes observes some discrete spots between the tyrosine sulfate spot and the origin that may have similar electrophoretic mobilities as small tyrosine sulfate-containing peptides. In addition, one usually finds, in particular with proteoglycans, a fairly continuous streak that reaches from the origin to and beyond the tyrosine sulfate spot. In extensive Pronase digests of protein samples containing both tyrosine sulfate and sulfated carbohydrate residues, this latter $^{35}SO_4$-labeled material can fog the tyrosine sulfate spot or, if present in excess over tyrosine sulfate, hide its presence (see Fig. 4D).

For reasons outlined above (see Section 1), [^{35}S]methionine and [^{35}S]cysteine may be present in proteins, along with sulfated residues, after $^{35}SO_4$ labeling in animals. Their presence poses no problem for the detection of tyrosine sulfate, since in the one-dimensional thin-layer electrophoresis tyrosine sulfate is well separated from methionine, cysteine, and also cystine, all of which remain near the origin. Cysteic acid migrates about 1.5 times as fast as tyrosine sulfate toward the anode at pH 3.5, being very close to serine sulfate (see below).

Determination of the Stoichiometry of Tyrosine Sulfation of Proteins

In determining the stoichiometry of tyrosine sulfation of proteins, the possibility of sulfation at multiple sites must be taken into account. For example, if a stoichiometry of 1 mol of tyrosine sulfate per mole of protein is found, this would be consistent with a single tyrosine residue being sulfated in all of the protein molecules, or with two tyrosine residues being sulfated in half of the protein molecules, and so on. Evidence for multiple-site sulfation of $^{35}SO_4$-labeled proteins separated in polyacrylamide gels can be obtained by "fingerprinting." The procedures used for fingerprinting are similar to those used in the study of phosphorylated proteins.[30,31] Gel pieces from one- or two-dimensional gels containing the protein of interest are incubated with trypsin or any other specific protease until this digestion is complete. The eluted sulfated peptides are then separated in two dimensions on cellulose thin-layer sheets by electrophoresis and ascending chromatography and detected by autoradiography (the conditions of electrophoresis and chromatography may have to be adapted for the sulfated protein under study). Individual sulfated peptides

[30] W. B. Huttner and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5402 (1979).
[31] W. B. Huttner, L. J. DeGennaro, and P. Greengard, *J. Biol. Chem.* **256,** 1482 (1981).

can be eluted from the cellulose with 50% pyridine and then used for hydrolysis and tyrosine sulfate analysis.

There are a number of possible approaches to determine the stoichiometry of tyrosine sulfation in individual sulfated proteins or in sulfated peptide fragments of these proteins. These include approaches based on the following considerations.

1. The direct chemical determination of tyrosine sulfate. This has the advantage that labeling of the protein of interest with radioactive isotopes is not required. However, the protein must be available in sufficient quantities to yield, after exhaustive alkaline hydrolysis, an amount of tyrosine sulfate that can be detected by amino acid analysis (about 10 pmol).[31a] With the advances made in the sensitivity of amino acid analysis, it should be feasible to develop methods for the determination of the stoichiometry of tyrosine sulfate in protein spots from two-dimensional polyacrylamide gels.

2. The determination of the proportion of tyrosine present as tyrosine sulfate. If the number of tyrosine residues in the protein under study is known, the stoichiometry of tyrosine sulfation can be determined as follows. Cells containing the protein are labeled with [^{3}H]tyrosine or double-labeled with $^{35}SO_4$ and [^{3}H]tyrosine (if the sulfate labeling is necessary to detect the protein in the subsequent steps). The protein is then obtained by immunoprecipitation or from two-dimensional gels (see Section 2) and subjected to exhaustive alkaline hydrolysis as described in Section 3. The residue of the neutralized supernatant (after step 6, Procedure III) is subjected to electrophoresis at pH 1.9 on cellulose thin-layer sheets (origin at 7 cm from the anodic edge of the sheet; 750 V for 40 min). Under this condition, tyrosine (migrating toward the cathode) is separated from tyrosine sulfate (migrating toward the anode). The tyrosine sulfate and the tyrosine spots, detected by ninhydrin staining of standard markers, are then counted for ^{3}H radioactivity. The values obtained (after correction for the recovery of internal standards) can be used to calculate the proportion of tyrosine that is present as tyrosine sulfate and, taking into account the moles of tyrosine per mole of protein, the moles of tyrosine sulfate per mole of protein. We have used this method to estimate the proportion of tyrosine sulfate to tyrosine in the total protein of PC12 cells and chicken embryo fibroblast, and have obtained values ranging from 0.2 to 0.5%. This suggests that tyrosine sulfate in proteins is far more abundant than tyrosine phosphate.

3. The determination of the specific activity of the $^{35}SO_4$ transferred to proteins. After prolonged labeling of cells with $^{35}SO_4$, including several changes of the medium containing the isotope, it is reasonable to assume

[31a] J. Dodt, H. P. Mueller, U. Seemueller, and J.-Y. Chang, *FEBS Lett.* **165,** 180 (1984).

that the specific activity of the $^{35}SO_4$ in the medium (which can be calculated) is similar to that of the intracellular sulfate donor and also to that of the sulfate bound to tyrosine residues of proteins. The ^{35}S radioactivity in the tyrosine sulfate spot generated from a known amount of the protein (see Sections 3 and 4) can in this case be used to estimate the stoichiometry of sulfation.

4. The use of purified tyrosylprotein sulfotransferase *in vitro*. An enzymatic activity catalyzing the sulfation of proteins on tyrosine residues, designated as tyrosylprotein sulfotransferase, has been described in cell lysates.[11] Once purified preparations of this enzyme are available, it should be possible to sulfate the protein of interest *in vitro* using 3′-phosphoadenosine-5′-phospho [^{35}S]sulfate of known specific activity, and thus to determine the stoichiometry of sulfation.

5. The determination of charge shifts in two-dimensional gels. If the unsulfated form of the protein of interest can be identified in two-dimensional polyacrylamide gels (e.g., by immunoblotting), it is possible to estimate the number of single charge shifts between the unsulfated form and the sulfated form(s) by the carbamoylation of these proteins.[32,33]

Concluding Remarks

Tyrosine sulfation of proteins is emerging as a widespread posttranslational modification in animals. The methods described in this chapter can be used to identify tyrosine-sulfated proteins and may help in studies on the role of these proteins in cell function. Is tyrosine sulfation the only case or just the first example of protein sulfation on amino acid residues? Do proteins undergo sulfation of serine and threonine residues? In preliminary experiments, in which extensive Pronase digests of $^{35}SO_4$-labeled rat brain proteins were analyzed by two-dimensional thin-layer electrophoresis and autoradiography, a radioactive substance with a mobility similar to that of serine *O*-sulfate was observed. However, it is too early at present for a definitive answer to the questions raised above. The study of protein sulfation on tyrosine and, possibly, on other amino acids is an area of research where little is known at present, but where new insights into cellular control mechanisms are likely to be gained in the future.

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[32] D. Bobb and B. H. J. Hofstee, *Anal. Biochem.* **40,** 209 (1971).
[33] R. A. Steinberg, P. H. O'Farrell, U. Friedrich, and P. Coffino, *Cell* **10,** 381 (1977).