Short Communications

The Formation of O-Sulphates of Serine and Threonine in Protein Hydrolysates

By K. MURRAY*

Medical Research Council Laboratory of Molecular Biology, Cambridge

(Received 14 August 1968)

Formation of sulphate esters of serine and threonine on hydrolysis of proteins in the presence of sulphate has been described by Moore (1963) and by Murray & Milstein (1967). The latter report showed that sulphate bound to basic residues of proteins could cause sufficient esterification of serine and threonine residues to introduce appreciable errors in amino acid analyses of hydrolysates of the protein. The stage at which esterification occurred remained uncertain, although the analogous formation of serine *O*-glutamate during the evaporation of protein hydrolysates (Ikawa & Snell, 1961) suggested that the reaction may have occurred during evaporation of hydrolysates rather than during hydrolysis of the proteins. Basic proteins (histones and protamines) are frequently prepared (and dried) as their sulphates. It is therefore very desirable to ascertain whether threonine and serine residues in these proteins are esterified during preparation of the proteins, or only during or after hydrolysis of the protein sulphates. Histones were extracted from chicken erythrocyte nuclei with ^{35}S -labelled H_2SO_4 to investigate the stage at which sulphate ester formation occurred.

Deoxyribonucleoprotein was prepared from pooled chicken blood and washed extensively with 0.14M-NaCl (Murray, Vidali & Neelin, 1968). The saline-washed pellet (13g. wet wt.) was stirred for 30 min. at 5° with 0.7N- H_2SO_4 (100ml.) containing about 22mg. of $\text{H}_2^{35}\text{SO}_4$ (about 3mc). The suspension was centrifuged (12000g for 10 min.) and four preparations of histone sulphate were made from the supernatant solution as follows:

(1) Dialysed against 100 vol. of cold water (six changes during 48 hr.), concentrated almost to dryness in a rotary evaporator (30°), diluted to 10 ml. with water and stored frozen.

(2) Dialysed as in (1) and freeze-dried.

(3) Treated with ethanol (2.5 vol.), and after 13 hr. at 5° the precipitate was harvested by centrifugation, washed twice with cold ethanol, dissolved in water (5 ml.) and stored frozen.

(4) Precipitated and washed with ethanol as

in (3), then washed with acetone and dried exhaustively in a vacuum desiccator.

Acid hydrolysates of each histone sulphate preparation were made by heating a solution (50 μl . of about 20 mg./ml.) with an equal volume of conc. HCl at 100° for 2 hr. in stoppered tubes. Reference samples of serine and threonine (7 mg./ml. in water) containing $\text{H}_2^{35}\text{SO}_4$ were treated similarly. Reactions were done in duplicate. Hydrolysates were either evaporated to dryness over NaOH pellets and conc. H_2SO_4 in a vacuum desiccator at room temperature (< 0.1 mm. Hg) and maintained under this vacuum for several hours, or evaporated to dryness over NaOH pellets and conc. H_2SO_4 in a desiccator at about 1 mm. Hg, and not maintained under vacuum. In the latter case more water was added (about 0.1 ml.) and the solution was again evaporated to dryness. This procedure (which was intended to remove HCl by distillation) was repeated several times. An additional hydrolysate (histone sulphate preparation 4) was concentrated almost to dryness, diluted with water, treated with excess of ammonia, and then dried and maintained under high vacuum overnight.

Tryptic digests of each histone sulphate preparation were made in 0.1M- NH_4HCO_3 by incubation at 37° for 22 hr. at an enzyme/substrate ratio about 1:80 by wt. Reaction mixtures were again dried over NaOH pellets and conc. H_2SO_4 at room temperature and maintained under high vacuum (< 0.1 mm. Hg) for several hours, or dried carefully (about 1 mm. Hg) with repeated additions of water, but not kept under vacuum when finally dried. An additional tryptic digest was prepared (histone preparation 1) and was acidified with 1 drop of 6N-HCl before evaporation to dryness; it was then kept under high vacuum for several hours.

The reaction mixtures were analysed by ionophoresis and radioautography (details in the caption to Fig. 1) and results are represented in Fig. 1. Serine *O*-sulphate was detected in histone hydrolysates that had been kept under high vacuum for a prolonged period (nos. 9–13, Fig. 1), but it was not found in hydrolysates that had been dried more gently (nos. 3–6, Fig. 1), nor was it found in a

* Present address: Department of Molecular Biology, University of Edinburgh.

Fig. 1. Results obtained by radioautography of reaction mixtures after ionophoresis. Nos. 1 and 2, acid hydrolysates of serine and threonine respectively, dried, but not maintained under high vacuum. Nos. 3-6, acid hydrolysates of histone sulphate preparations 1-4 respectively, treated similarly. Nos. 7 and 8, acid hydrolysates of serine and threonine respectively, dried and maintained under high vacuum. Nos. 9-12, acid hydrolysates of histone sulphate preparations 1-4 respectively, treated similarly. No. 13, acid hydrolysate of histone hydrochloride (from another experiment) in the presence of $H_2^{35}SO_4$, dried and maintained under high vacuum. No. 14, the same as no. 12, but neutralized with ammonia before drying. No. 15, tryptic digest of histone sulphate preparation 1, dried, but not maintained under high vacuum. No. 16, the same preparation as no. 15, but acidified before drying and then maintained under high vacuum. Nos. 17-20, tryptic digests of histone sulphate preparations 1-4 respectively, dried and maintained under high vacuum. All the samples were dissolved in water (0.05 ml.) for ionophoresis on Whatman 3MM paper in pyridine-acetate buffer, pH 3.5 (Ambler, 1963). Radioautographs of dried papers were made with Kodak Blue Brand X-ray film developed in Kodak DX 80 developer. Abbreviations: ThrS, threonine *O*-sulphate; SerS, serine *O*-sulphate.

hydrolysate that had been neutralized before drying (no. 14, Fig. 1). This result was obtained regardless of the preparative history of the histone sample. The amount of $H_2^{35}SO_4$ added to the reference samples of serine and threonine greatly exceeded that present in the histone samples, so that detection of ester formation was much more sensitive in these instances, but here also (comparing nos. 1 and 2 with nos. 7 and 8, Fig. 1) esterification was very much more pronounced after prolonged drying under high vacuum. The large amount of radioactive sulphate used in these particular samples revealed the formation of smaller quantities of several other reaction products, none of which has been identified (nos. 7 and 8, Fig. 1). None of the tryptic digests dried directly after digestion provided any evidence of the formation of sulphate esters, for in each case radioactivity was associated only with inorganic sulphate (nos. 17-20, Fig. 1). However, when a tryptic digest of histone sulphate (preparation 1; at no time had this been subjected to drying) was acidified before drying and then kept under high vacuum for several hours, it appeared that some sulphate ester formation occurred, since

the digest now contained a small quantity of radioactive substances that were neutral or cationic at pH 3.5, as well as traces of acidic radioactive substances (no. 16, Fig. 1). Control samples of the four histone sulphate preparations were examined in the same way as their hydrolysates. In each sample, radioactivity was found only in the position of inorganic sulphate, which proves that serine and threonine residues in the histones were not esterified during any of the preparative procedures.

These results show that formation of *O*-sulphates of serine and threonine residues in hydrolysates or digests of histone sulphates (and presumably other proteins or peptides containing sulphate) occurs only during rather drastic drying of acidic samples. There was no evidence of similar ester formation during the preparation, including drying, of histone sulphates.

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