

## Esters of Serine and Threonine in Hydrolysates of Histones and Protamines, and Attendant Errors in Amino Acid Analyses of Proteins

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1. Partial acid hydrolysates of histones from various origins and of protamine were analysed by a two-dimensional ionophoretic procedure to reveal strongly acidic ninhydrin-positive components. 2. Histone fractions prepared by extraction with sulphuric acid gave rise to spots identified as serine *O*-sulphate and threonine *O*-sulphate. These two compounds, which were not found in hydrolysates of corresponding fractions prepared by extraction with hydrochloric acid, were artifacts. 3. Hydrolysis of proteins in the presence of traces of sulphate can lead to the formation of the *O*-sulphates of serine and threonine. This can cause errors, which may sometimes be serious, in amino acid analyses of proteins. 4. *O*-Phosphoserine was obtained in small amounts from some histone fractions and from protamine, but was undetectable in other histone fractions, notably those of lower lysine content.

Ord & Stocken (1966*a,b*) have reported the presence of *O*-phosphoserine in rat histones. This observation prompted a search for *O*-phosphoserine in a number of histone preparations and their chromatographic fractions, and also in some protamines. In the work described in the present paper a two-dimensional ionophoretic procedure was used to survey partial acid hydrolysates of these proteins. In addition to *O*-phosphoserine, this method revealed the presence of two other components. These two substances were shown to be the *O*-sulphates of serine and threonine, which resulted from acid hydrolysis of the proteins in the presence of trace amounts of sulphate. This reaction, which had been observed also by Moore (1963), can lead to substantial errors in amino acid analyses of proteins. *O*-Phosphoserine was found in certain histone fractions, but not in others; it was present also in protamine and was not an artifact of the analytical operations.

### EXPERIMENTAL

*Histone and protamine preparations.* Commercial samples of salmine and clupeine were used. Dr A. E. Mirsky kindly provided a preparation of purified protamine that contained no tyrosine. Histones from calf and rabbit thymus were prepared by H<sub>2</sub>SO<sub>4</sub> extraction and fractionated by chromatography on Amberlite IRC-50 with buffered guanidinium chloride solutions, as described by Rasmussen, Murray & Luck (1962). Histone fractions of chicken-erythrocyte nucleoprotein, washed with 0.9%

NaCl, were prepared by titration to the appropriate pH with H<sub>2</sub>SO<sub>4</sub> (Murray, Vidali & Neelin, 1966) and subsequent precipitation with ethanol. Dr J. Hindley kindly supplied samples of calf-thymus histone fractions f1, f2a, f2b and f3 (Johns & Butler, 1962).

*Hydrolyses and amino acid analyses.* Samples of the proteins (10 mg./ml.) in 6*N*-HCl were heated in tubes stoppered with glass marbles in a boiling-water bath for periods of up to 3 hr. The usual period used for partial hydrolysis for the detection of *O*-phosphoserine was 65 min. Hydrolyses for periods of over 3 hr. were carried out in evacuated sealed tubes, which were heated in an oven at 105°. All hydrolysates were dried under vacuum (Edwards Hyvac oil pump) over NaOH, and dissolved in water for ionophoretic analysis, or in 0.2*M*-sodium citrate buffer, pH 2.2, for analysis on an automatic amino acid analyser (Spackman, Stein & Moore, 1958). Solutions of serine or threonine in 6*N*-HCl were heated with various quantities of H<sub>2</sub>SO<sub>4</sub> or H<sub>3</sub>PO<sub>4</sub> in evacuated sealed tubes at 105° for 20 hr. The hydrolysates were dried and dissolved as described above for amino acid analysis.

*Ionophoretic analysis.* Details of the apparatus and the composition of the pyridine-acetate buffers were as described by Ambler (1963). Aqueous solutions of the protein hydrolysates, equivalent to about 10 mg. of protein, were applied as 8 cm. streaks 10 cm. from the end of a sheet of Whatman no. 3MM paper. Spots of red Fybriter ink (Conway Stuart Pen Co. Ltd.) and of *O*-phosphoserine were included as markers. After ionophoresis at pH 6.5 and 60 v/cm. for 50 min., a 20 cm. strip was cut from each sample to cover 10 cm. at either side of the *O*-phosphoserine as indicated by the markers. The strips were sewn on to new sheets of Whatman no. 3MM paper and subjected to ionophoresis at pH 3.5 and 60 v/cm. for 1½ hr. at right angles to the direction of the first ionophoresis. The same markers

were used as before. The dried papers were then dipped in 0.2% ninhydrin in acetone. Ionophoretic analyses for serine or threonine were carried out at pH 2 and 120 v/cm. for 15 min. and stained with 0.2% ninhydrin-5% collidine in acetone.

**Hydrolysis with phosphatase.** Reactions were carried out on paper by careful application of a solution of bacterial alkaline phosphatase (1 mg./ml.) (Pigretti & Milstein, 1965) in 0.1 M-NH<sub>4</sub>HCO<sub>3</sub> solution, pH 8.5 (C. P. Milstein, unpublished work). The moist paper was incubated in a humid atmosphere for 1 hr. at 37°. After being dried the paper was sewn to another sheet of paper for ionophoretic analysis at pH 2.

**Neutron activation analysis for phosphorus.** A section of paper that was believed to contain *O*-phosphoserine was irradiated with thermal neutrons in the reactor HERALD at the Atomic Weapons Research Establishment, Alder-

maston, Berks., through the kindness of Dr D. H. Brown (Murray & Offord, 1966). After detection of the appropriate area by radioautography, the relevant circle of paper was cut out and stuck to an aluminium planchet. The half-life of the radioactive substance was obtained from counts made daily for about 3 weeks with a gas-flow counter (Nuclear-Chicago Corp.).

**Mass spectra of sulphate esters.** Mass spectra were obtained with an MS9 mass spectrometer (Associated Electrical Industries Ltd.); samples were inserted directly into the ion source. We are greatly indebted to Dr D. H. Williams for these spectra.

## RESULTS

The ionophoretic analyses of partial hydrolysates of histones and protamines are illustrated in Fig. 1.

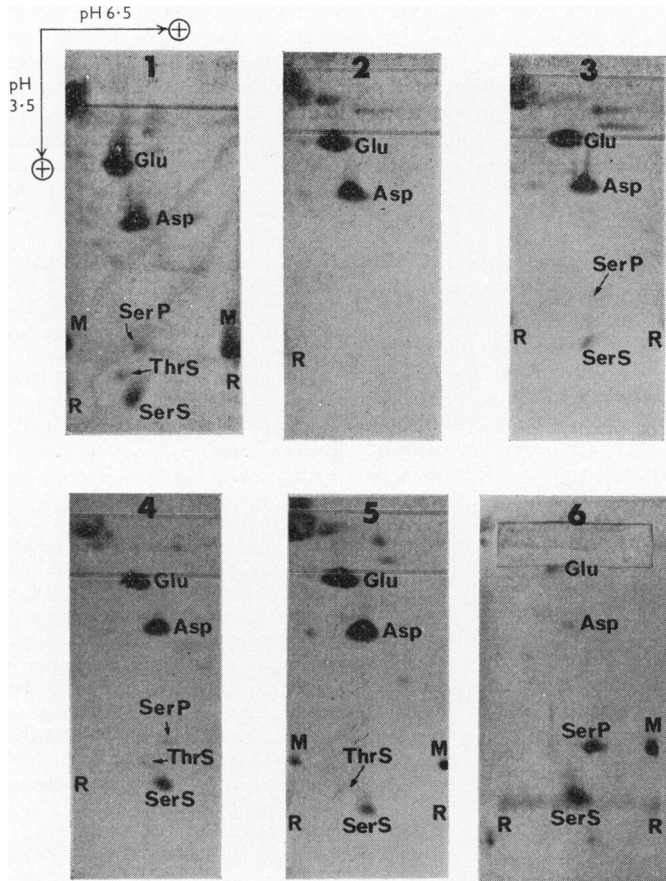


Fig. 1. Analyses of partial hydrolysates (1 hr. at 105° in 6N-HCl) of histone fractions and protamine. Ionophoresis was carried out first at pH 6.5; strips containing acidic amino acids were cut out and stitched to a second piece of Whatman no. 3MM paper for ionophoresis at pH 3.5 in a direction at right angles to that of the first ionophoresis. The papers were stained with ninhydrin. SerP, *O*-phosphoserine; SerS, serine *O*-sulphate; ThrS, threonine *O*-sulphate; M, *O*-phosphoserine reference; R, red ink reference. The samples were: (1) calf-thymus histone fraction Ib; (2) rabbit-thymus histone fraction IIb; (3) calf-thymus histone fractions III and IV; (4) chicken-erythrocyte histone extracted at pH 2.15 (this contains fraction Ib); (5) chicken-erythrocyte histone extracted at pH 1.38 after prior extraction at pH 1.78 (this is equivalent to fraction IIb); (6) protamine sulphate.

Table 1. *Qualitative analyses of esters of serine and threonine in acid hydrolysates of various histone and protamine preparations*

Histone fractions were the chromatographic fractions described by Rasmussen *et al.* (1962), the fractions described by Johns & Butler (1962), or those obtained directly by titration with  $H_2SO_4$  (Murray *et al.* 1966). Hydrolysates were analysed as shown in Fig. 1. Hydrolyses were in 6N-HCl at 105°, and analyses were as described in the Experimental section.

Protein	Time of hydrolysis (hr.)	O-Phosphoserine	Serine O-sulphate	Threonine O-sulphate
Calf-thymus histone fraction Ib	1	+	+++	+
Calf-thymus histone fraction IIb	1	+	—	—
Calf-thymus histone fractions III and IV	1	Trace	+	—
Calf-thymus histone mixed fractions f1, f2a, f2b and f3	1	+	—	—
Rabbit-thymus histone fraction IIb	1	—	—	—
Chicken-erythrocyte histone extracted at pH 2.15	1	+	>++++	++
Chicken-erythrocyte histone extracted at pH 1.9	1	+	>++++	++
Chicken-erythrocyte histone extracted at pH 1.38	1	Trace	>++++	++
Chicken-erythrocyte histone extracted at pH 1.02	1	—	+++	+
Chicken-erythrocyte histone dissociated with NaCl as above, but with 1 equiv. of $H_2SO_4$	1	+	—	—
	18		++++	++
Protamine (commercial)	1	+++	++++	—
Protamine (from A. E. Mirsky)	1	++	++++	—

A ninhydrin-positive substance having the same mobilities at pH 3.5 and pH 6.5 as the *O*-phosphoserine marker was present in calf-thymus histone fractions Ib and IIb (Rasmussen *et al.* 1962), in the corresponding fractions of chicken-erythrocyte histone and in the histone fraction unique to chicken erythrocytes (fraction 5; Neelin, 1964). The substance was not detected in histone fraction IIb of rabbit thymus, and was present only in trace amounts in the corresponding fraction of chicken erythrocytes and in calf-thymus histone fraction III (Table 1). Commercial preparations of protamine sulphate and a purified sample of protamine (free from tyrosine; kindly provided by Dr A. E. Mirsky) contained greater quantities of the substance than did any of the histone fractions examined (Table 1). The substance was shown to be *O*-phosphoserine by several criteria in addition to its ionophoretic mobility. It emerged in the same position as authentic *O*-phosphoserine and cysteic acid on the amino acid analyser. After total hydrolysis for 20 hr. in 5.7N-hydrochloric acid, it gave a single peak in the position of serine on the amino acid analyser. Treatment with alkaline phosphatase gave serine. Neutron activation analysis resulted in the formation of a spot of radioactivity that had a half-life of 14.5 days (the half-life of  $^{32}P$  is 14.3 days).

Two ninhydrin-positive substances that at pH 3.5 had even higher mobility than *O*-phosphoserine (Fig. 1) were obtained in several instances. Only the faster-moving one was found in the protamine hydrolysates. On acid hydrolysis for 20 hr. in 5.7N-hydrochloric acid at 105°, the faster-moving

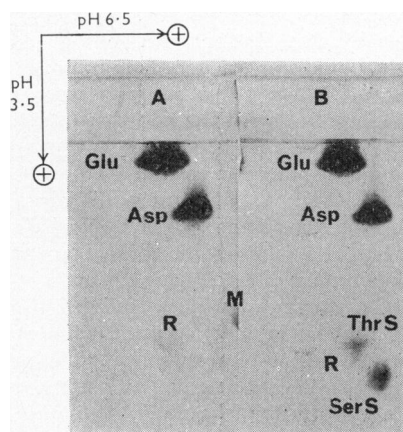


Fig. 2. Ionophoretic analyses of hydrolysates (21 hr. at 105° in 6N-HCl) of chicken-erythrocyte histones prepared by dissociation by 2M-NaCl from nucleoprotein washed with 0.9% NaCl. The analyses were carried out as described in the legend to Fig. 1, and the symbols have the same significance. The samples were: (A) histone hydrolysed in the usual way; (B) histone hydrolysed in the presence of  $H_2SO_4$  (equivalent in quantity to the basic amino acids of the protein).

substance gave only serine and the other gave only threonine, but neither was hydrolysed by phosphatase. Dr J. B. Clegg drew our attention to a paper by Moore (1963), reporting the formation of serine *O*-sulphate when proteins were hydrolysed in the presence of relatively large quantities of sodium sulphate. The protamine and histone

Table 2. *Formation of sulphates of serine and threonine under conditions of acid hydrolysis in the presence of sulphuric acid*

Conditions were as described in the Experimental section. Values for serine *O*-sulphate and threonine *O*-sulphate are moles/100 moles of the total amino acids recovered.

Concn. of serine ( $\mu$ moles/ml. in 6N-HCl)	Molar ratio H <sub>2</sub> SO <sub>4</sub> /serine	Serine <i>O</i> -sulphate (%)	Concn. of threonine ( $\mu$ moles/ml. in 6N-HCl)	Molar ratio H <sub>2</sub> SO <sub>4</sub> /threonine	Threonine <i>O</i> -sulphate (%)
100	0.9	2.8	100	0.9	0.7
20	0.9	1.3	20	0.9	
100	4.7	5.4	100	4.7	2.5
20	4.7	6.1	20	4.7	2.3
5	4.7	5.1	5	4.7	2.5
1	4.7	7.6	1	4.7	5.1
0.1	4.7	5.5	0.1	4.7	

samples examined had indeed been sulphates. However, no trace of the two fast-moving substances was obtained from the histones of the chicken-erythrocyte nuclei extracted with hydrochloric acid, nor from a mixture of the histone fractions f1, f2a, f2b and f3 described by Johns & Butler (1962). Neither were they found in hydrolysates of histones prepared from chicken-erythrocyte nuclei by dissociation with sodium chloride. In contrast, *O*-phosphoserine was found in all these hydrolysates. When these samples were hydrolysed as before, but with the addition of sulphuric acid (1 equiv./mole of basic amino acids), the two fast-moving substances were found on subsequent ionophoresis (Fig. 2). Samples of serine and threonine gave rise to the two characteristic spots when heated in 6N-hydrochloric acid at 105° for 18 hr. with 1 equiv. of sulphuric acid/mole of serine or threonine. Both substances emerged from the automatic amino acid analyser in the same position as cysteic acid and *O*-phosphoserine. The product of the reaction with serine gave only serine on acid hydrolysis, and similarly only threonine was obtained from the product of the reaction with threonine. That the two substances were in fact the *O*-sulphates of serine and threonine was confirmed when very large peaks at 64 mass units (attributable to sulphur dioxide) were found in their mass spectra.

The quantity of the *O*-sulphates of serine and threonine in a hydrolysate of a histone sulphate preparation appeared to increase with time of hydrolysis. However, the isolated serine and threonine sulphates were readily and completely converted back into serine or threonine by 6N-hydrochloric acid at 105°. Formation of the sulphates was studied by heating serine and threonine under these conditions for 20 hr. at various concentrations and with two different quantities of sulphuric acid. Analyses of these reaction mixtures are in Table 2. Formation of the

ester was not favoured in any of these cases. It is not clear from these experiments whether esterification occurred principally during 'hydrolysis' or during subsequent drying of the sample *in vacuo* (as has been suggested by Dr T. Langan, unpublished work). Similar experiments with phosphoric acid showed that *O*-phosphoserine was not produced in an analogous way.

#### DISCUSSION

The qualitative analyses of partial hydrolysates of histones that are given in Table 1 and Fig. 1 extend the findings of *O*-phosphoserine by Ord & Stocken (1966a,b) in histones of rat liver and rat thymus to materials from other sources. Most, but not all, of the histone fractions contained a very small quantity of *O*-phosphoserine. The qualitative analysis was selected partly because of its simplicity and sensitivity, but partly also because the marked facility of histones for binding phosphate and the ability to acid hydrolysis of *O*-phosphoserine combine to make a meaningful determination of this amino acid a difficult proposition. However, rough estimates are possible if one assumes that after 60 min. of partial acid hydrolysis about 20% of the phosphate is recovered as *O*-phosphoserine. This value is derived from studies of the release of *O*-phosphoserine during acid hydrolysis in phosphoglucomutase (Milstein & Sanger, 1961) and in alkaline phosphatase (Milstein, 1964). It seems that not more, and probably much less, than 1 mole of phosphate is covalently bound/mole of histone, even in the fraction with the highest *O*-phosphoserine content. This conclusion is suggested by the fact that in no case did the intensity of the spot (stained with ninhydrin) obtained with 0.5  $\mu$ mole of protein (assuming mol.wt. 20000) approach that produced by 0.1  $\mu$ mole of *O*-phosphoserine. It was most abundant in the histone fractions of highest lysine content, and in fraction 5

of chicken-erythrocyte histones, which is also known to have a high serine content (Neelin, 1964). The histone fractions of relatively high arginine content (the chromatographic fraction III and the pH 1.02 extract) contained less, or no, *O*-phosphoserine. The two protamine samples, however, contained appreciable quantities of *O*-phosphoserine, although it may be very significant that Dr Mirsky's purified preparation contained less than the commercial sample.

*O*-Phosphothreonine was not detected in any of the partial acid hydrolysates. This amino acid has a slightly lower mobility than *O*-phosphoserine at pH 3.5. However, quantities less than about one-fifth of that of *O*-phosphoserine would not have been detected in these experiments.

The sulphate esters of serine and threonine that were found in hydrolysates of the histone sulphates were artifacts formed during hydrolysis or subsequent drying, but *O*-phosphoserine was not formed in a corresponding way. It may be relevant to recall the formation of serine *O*-glutamate under somewhat similar circumstances (Ikawa & Snell, 1961); in this case it was clearly shown that esterification occurred principally during the evaporation of the hydrolysates, but not appreciably during the hydrolysis itself. Experiments in which samples of serine or threonine in dilute sulphuric acid were evaporated to dryness *in vacuo* and then examined on the amino acid analyser, or were applied directly to the analyser without prior evaporation, were inconclusive because the relatively large quantities of sulphuric acid in the samples led to badly distorted chromatograms. Apparently the sulphate ions bound as counter-ions to the basic amino acids are quite sufficient to promote the ester formation. With the protamines, where about two-thirds of the molecule is arginine, formation of serine *O*-sulphate is even more pronounced.

It is noteworthy that serine *O*-sulphate and threonine *O*-sulphate were found in hydrolysates of chromatographic fraction Ib of calf-thymus histone. This fraction (in common with others) had been prepared from histone extracted with sulphuric acid, but chromatographed on a column of Amberlite IRC-50 with quite concentrated solutions (8–11%, w/v) of guanidinium chloride and then dialysed extensively against water. In spite of this treatment, sufficient sulphate remained bound to the protein to result in ester formation on hydrolysis. The other chromatographic fractions did not behave similarly, however, which suggests that the high lysine content, or rather the distribu-

tion of lysine residues, may be responsible for the very tight binding of sulphate.

Serine *O*-sulphate and threonine *O*-sulphate are formed in the presence of even very small quantities of sulphate. This could lead to serious errors in amino acid analyses by lowering the yield of serine and threonine and by increasing the quantity of the breakthrough material, usually computed as cysteic acid; an unusually high value for this amino acid could, in fact, provide a useful indication that some ester formation had occurred. Our colleague Dr A. G. Weeds drew our attention to the fact that ammonium sulphate, so popular a reagent in protein preparations, may bind sufficiently firmly to some proteins to contribute precisely this analytical error. A. G. Weeds (unpublished work) has, in fact, obtained remarkably low values for serine and threonine and an almost exactly correspondingly high value for cysteic acid in analyses of some myosin preparations that had been made by methods involving ammonium sulphate precipitation followed by extensive dialysis. It is noteworthy that myosin has a high lysine content and, like histone fractions Ia and Ib, a very irregular distribution of lysine residues.

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