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Hydrolysis of Phosphopeptides

I. Acid Hydrolysis of O-Phosphoryl-DL-serylglycine and Glycyl-(O-phosphoryl)-DL-serine. Sequence Inversion in Dilute Hydrochloric Acid

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The hydrolysis of SerP-Gly * and Gly-SerP in 2 N hydrochloric acid at 100° and in conc. hydrochloric acid at 37° was studied by column chromatographic separation of the hydrolysis products on an anion exchange resin. In 2 N hydrochloric acid sequence inversion occurs with both phosphopeptides. Gly-SerP is hydrolysed faster than SerP-Gly in both conc. and 2 N acid and hydrolysis takes place almost entirely at the peptide bond. SerP-Gly is largely dephosphorylated to serylglycine.

Partial acid hydrolysis is the general method used for breaking down peptide chains to di- and tripeptide fragments. In the large amount of work done during the last deecade on the determination of amino acid sequences in poly-peptides and proteins, the conditions of hydrolysis, *e.g.* temperature, time and acid strength, have been varied widely. It is known, however, that these variations can have a marked effect on the results of hydrolysis [1–4]. In the partial hydrolysis of phosphoproteins and phosphorylated polypeptides, different conditions of hydrolysis may cause still greater differences in results, since in these materials both peptide and phosphate ester bonds may be hydrolysed.

Several investigations have been reported on amino acid sequences near O-phosphorylserine residues in phosphoproteins such as casein, phosvitin, ovalbumin and pepsin [5–8]. A large amount of work has also been done on the determination of the structure around the "*active centers*" of enzymes such as trypsin, chymotrypsin, horse-liver ali esterase and phosphoglucomutase, using the technique of deactivation of the enzyme by a phosphorylating reagent, partial acid hydrolysis of the product and separation and determina-

* The following abbreviations are used here: SerP, O-phosphorylserine; SerP-Gly, O-phosphorylserylglycine; Gly-SerP, glycyl-(O-phosphoryl)-serine.

tion of the structure of the resulting peptides containing O-phosphorylserine [4,9–14].

In these investigations different concentrations of hydrochloric acid have been used for the partial hydrolysis, and it therefore appeared essential to know more about the hydrolysis of phosphorylated peptides, especially in view of the observations of Sanger and Thompson [2] and Schaffer *et al* [4], that sequence inversion may take place under certain conditions. Furthermore, a knowledge of the hydrolytic behaviour of phosphorylated peptides might assist an understanding of the action of proteolytic enzymes and phosphatases [15] on them. In the present investigation a study was therefore made of the hydrolysis of the two model peptides SerP-Gly and Gly-SerP in conc. hydrochloric acid at 37° and in 2 N hydrochloric acid at 100°.

EXPERIMENTAL

Materials

The *O-phosphoryl-*DL*-serylglycine* (SerP-Gly) and *glycyl-(O-phosphoryl)-*DL*-serine* (Gly-SerP) were samples of materials, of which the syntheses and analytical data have been described previously [16,17]. Their purity was further demonstrated by the column chromatographic results described below (Fig. 1, zero time hydrolysis).

Hydrolysis

A. In 2 N HCl: About 10 mg of phosphopeptide was dissolved in 1 ml of 2.02 N HCl (3 × glass-distilled azeotropic acid, diluted with distilled water), and 0.1 ml portions of the solution were hydrolysed in sealed tubes of 4 × 0.6 cm. Hydrolyses for 0.5 h and 1.0 h were done in boiling water, those for longer times in an oven at 100° ± 1°. After hydrolysis the tubes were immediately placed in an ice-box at −20° and kept at this temperature until just before the chromatographic analysis, when they were opened and diluted with 2 ml of water.

B. In conc. HCl: The hydrolyses in conc. HCl (Merck p.a.) were done essentially in the same way, but in an air thermostat at 37 ± 0.5°C. The sealed tubes containing the hydrolysates were kept at −20° until just before chromatography. The tube was then opened and the hydrochloric acid was removed *in vacuo* over NaOH. The dry residue was dissolved in 2 ml of water.

Chromatography

The method for the separation of phosphorylated peptides that has been described in a previous paper [18] from this laboratory was used. As only three phosphorus-containing compounds, SerP, SerP-Gly and Gly-SerP had to be separated, it was possible to decrease the height of the column and increase the solvent gradient. A column of 1.2 cm diameter was packed to a height of 20 cm with Dowex 1-X2 resin in formate form. The hydrolysate was applied and the column was eluted with 0.1 M formic acid. After 24 ml, gradient elution was started using 1.0 M pyridinium formate, pH 4.5, in the reservoir. The mixing chamber volume was 550 ml.

Fractions of 2 ml were collected in 180 × 18 mm calibrated test tubes. The flow rate was 24 ml/h, and a complete separation took 10 h. The fractions were analysed according to Moore and Stein [19] by addition of 1 ml of ninhydrin solution, dilution with 10 ml of ethanol-water (1:1) and measurement of the optical density at 575 mμ using a Bausch & Lomb monochromatic colorimeter. The peaks found were identified by their chromatographic behaviour (*cf.* Fig. 1) and by N-terminal analysis (see below). The results are shown in Tables 1 and 2.

Ninhydrin colour values. A value of 1.03 has been reported [7] for O-phosphorylserine. The colour values for SerP-Gly and Gly-SerP were determined as follows. Samples of

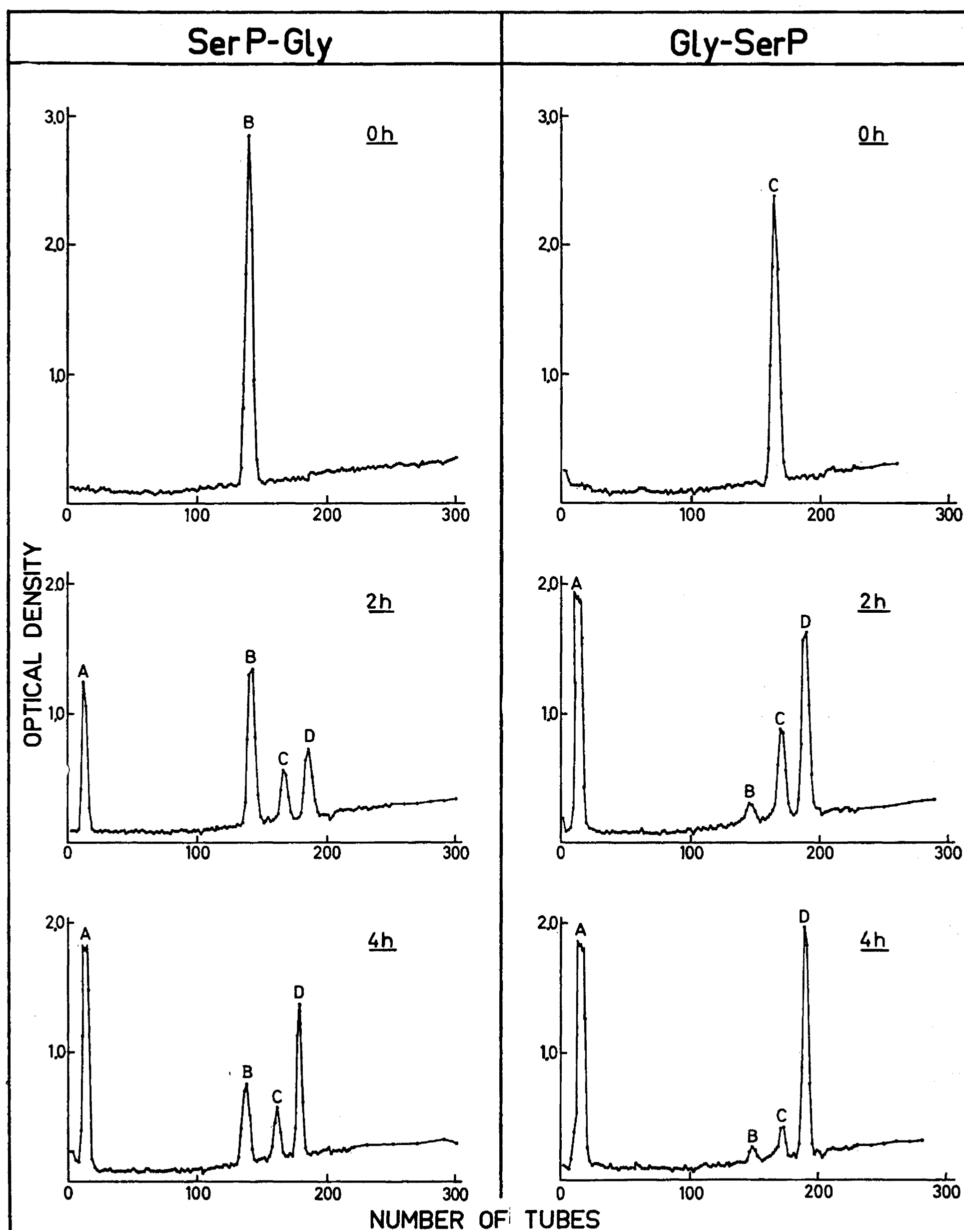


Fig. 1. Column chromatographic separation of the products of hydrolysis of SerP-Gly and Gly-SerP in 2 N HCl, 100°. Peak A contains the unphosphorylated amino acids and peptides; B is SerP-Gly; C is Gly-SerP and D is SerP.

these two compounds and of leucine were dried for 3 h at 70° *in vacuo* over P_2O_5 and 3—5 mg of each compound was dissolved in 50 ml of 0.2 M sodium citrate buffer, pH 5.0. One ml of these solutions was used for the ninhydrin reaction, and the optical density was measured with a Beckman B spectrophotometer at 570 mμ. The colour values obtained were: SerP-Gly 1.00 ± 0.02; Gly-SerP 0.98 ± 0.03.

Identification of peak B obtained from Gly-SerP as SerP-Gly by N-terminal analysis *. Fifty mg of Gly-SerP was hydrolysed for 2 h at 100° in 5 ml of 2 N HCl. After evaporation over NaOH *in vacuo* the hydrolysate was chromatographed as described above. Fractions of 2 ml were collected and 0.2 ml of each was used for ninhydrin reaction. The fractions containing peaks A and B (Fig. 1) were evaporated to dryness *in vacuo* (peak A was used for the complete hydrolysis experiment described below).

Peak B, from its position in the chromatogram was suspected to be SerP-Gly. Its N-terminal amino acid was identified by the DNP-method of Sanger as described by Janz *et al.*[13] The sample, weighing about 3 mg, was shaken for 3 h at 40° with 2 ml of 66 % ethanol containing 1 % $NaHCO_3$ and 1 % 1-fluoro-2,4-dinitrobenzene. After evaporation to dryness, the mixture was dissolved in 4 ml of water, acidified and extracted with 3 × 2 ml of ether to remove fluorodinitrobenzene and dinitrophenol. The DNP derivative of B was extracted from the water phase with 3 × 2 ml of methyl acetate and the combined extract was evporated to dryness and hydrolysed for 2 h at 100° with 2 ml of 2 N HCl. The hydrolysate was diluted with 1 ml of water and the DNP-amino acid was taken up in 3 × 2 ml of methyl acetate and identified as N-dinitrophenyl-(O-phosphoryl)-DL-serine (DNP-SerP) by paper chromatography in the solvent systems *tert*-amyl alcohol — diphthalate buffer and 1.5 M phosphate buffer, pH 6.0. Synthetic DNP-SerP prepared ** from SerP and fluorodinitrobenzene was used as reference, it had an R_F value of about 0.7 in the phosphate buffer. In the *tert*-amyl alcohol — diphthalate system, this compound had moved only 2 cm after 100 h running.

Authentic SerP-Gly, treated as peak B, gave exactly the same results.

Complete hydrolysis of peak A obtained from SerP-Gly and Gly-SerP. To determine the amount of peptide in the dephosphorylated fraction obtained from Gly-SerP, peak A from the above chromatogram was dissolved in 3 ml of water. Two samples of 1 ml were evaporated to dryness, and one of them was hydrolysed for 20 h at 110° in 0.1 ml of 6 N HCl and again evaporated to dryness. The two samples were then dissolved separately in 0.35 M sodium citrate buffer (5 ml), pH 5.28, and 2 ml of each solution was used for reaction with ninhydrin solution. The ninhydrin colour yields were:

Unhydrolysed peak A from Gly-SerP 1.88
Hydrolysed » » » » 1.96

This increase of 4 % in the ninhydrin colour is equal to about 7 % by weight of peptide in peak A.

From a 2 h hydrolysate of SerP-Gly, peak A when isolated and treated as above, gave the following ninhydrin colour yields:

Unhydrolysed peak A from SerP-Gly 0.913
Hydrolysed » » » » 1.177

Complete hydrolysis of this fraction from SerP-Gly gave an increase of 30 % in ninhydrin colour, indicating that the fraction contained about 45 % by weight of dephosphorylated peptide.

RESULTS

The rates of hydrolysis of SerP-Gly and Gly-SerP in 2 N HCl at 100° and in conc. HCl at 37° are shown in Fig. 2 as measured by the ninhydrin colour

* Schaffer *et al.*[4], by a similar procedure have demonstrated the formation of Gly-SerP from SerP-Gly in 2N HCl.

** The preparation of DNP-SerP was made by Bo Öberg in this laboratory.

Table 1. Hydrolysis of 1 mg of phosphopeptide in 0.1 ml of 2.0 N HCl at 100°.

Hours	Ninhydrin colour							
	1.0 mg SerPGly				1.1 mg GlySerP			
	Unphosphorylated	SerPGly	GlySerP	SerP	Unphosphorylated	SerPGly	GlySerP	SerP
0	0	8.01	0	0	0	0	7.46	0
0.5	0.56	(5.77)	0.36	0.30	1.58	0.15	5.17	1.47
1	1.48	5.64	0.76	0.84	3.36	0.40	4.86	2.75
2	2.59	4.07	1.23	1.62	6.52	0.43	2.28	4.64
4	5.90	1.93	1.03	2.75	9.55	0.31	0.72	4.82
6	7.61	0.96	0.49	3.48				
20	13.25	0	0	1.70				

Table 2. Hydrolysis of 1 mg of phosphopeptide in 0.1 ml of conc. HCl at 37°

Days	Ninhydrin colour							
	1.0 mg SerPGly				1.1 mg GlySerP			
	Unphosphorylated	SerPGly	GlySerP	SerP	Unphosphorylated	SerPGly	GlySerP	SerP
0	0	7.19	0	0	0	0	7.01	0
1	0.67	6.32	0	0.51	2.72	0	4.92	2.46
3	1.62	5.12	0	1.13	5.10	0	2.13	4.64
7	2.85	3.68	0	1.89	7.92	0	0.55	6.45

yield from chromatographic fractions of hydrolysates. Fig. 2 also shows the formation of a mixture of unphosphorylated amino acids and peptides (peak A), SerP (peak D) and the formation of phosphopeptide with an *inversed* amino acid sequence during hydrolysis in 2 N HCl.

The hydrolysis of Gly-SerP in 2 N HCl is appreciably faster than that of SerP-Gly, as can be seen from Fig. 2 and the following table.

Time	0	0.5	1	2	4	6	20 h
SerP-Gly	0	(27.9)	29.5	49.1	75.9 %	88	100 % hydrolysis
Gly-SerP	0	30.6	35.0	69.4	90.3 %	—	— »

In spite of the faster hydrolysis of Gly-SerP the amount of this compound formed by sequence inversion of SerP-Gly, is more than twice as large as the amount of SerP-Gly formed from Gly-SerP.

Fig. 2 also shows, that much more SerP is formed from Gly-SerP than from SerP-Gly, and that comparatively more ninhydrin colour is obtained from the dephosphorylated peaks (A) from Gly-SerP than from SerP-Gly. Since all the compounds concerned have ninhydrin colour values of 100 ± 0.05 (relative to leucine), calculation shows that peak A from Gly-SerP contains almost only the free amino acids (glycine and serine) while peak A from SerP-Gly contains large amount of dephosphorylated peptide (serylglycine) *. This was confirmed by isolation of peak A, followed by complete hydrolysis. These results show that Gly-SerP is hydrolysed mainly at the peptide bond yielding glycine and SerP, while SerP-Gly, apart from some hydrolysis at the peptide bond, to a large extent releases its phosphoric acid residue yielding serylglycine.

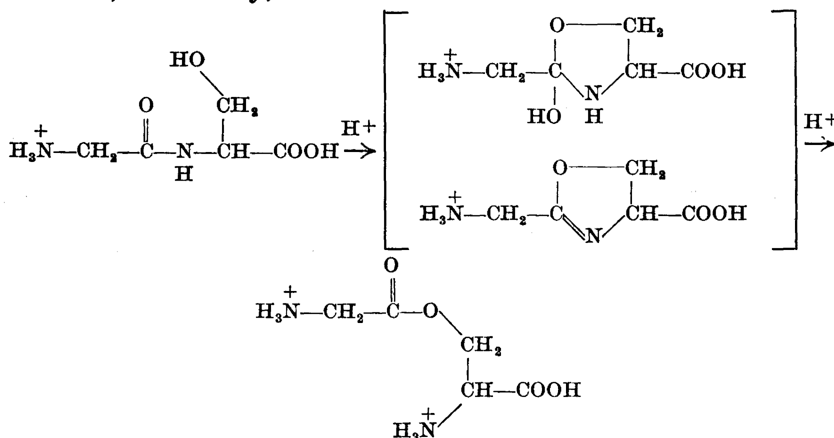
In conc. HCl, Gly-SerP is hydrolysed much faster than SerP-Gly:

Time	0	1	3	7	days
SerP-Gly	0	12.2	28.9	48.9	% hydrolysis
Gly-SerP	0	29.7	69.2	92.2	% »

Here also Gly-SerP is hydrolysed mainly at the peptide bond, while SerP-Gly affords a large amount of dephosphorylated peptide, as can be calculated from the colour yield. No sequence inversion was observed in conc. HCl.

DISCUSSION

Peptide bonds involving the amino groups of serine and threonine residues with their β-hydroxyl groups intact are known to be unusually labile to acid hydrolysis [1,20]. This lability is a consequence of an N→O acyl shift *via* an oxazolin [21] or, more likely, a hemiacetal [22] derivative:



* Glycylserine may be formed by sequence inversion, but should be hydrolysed very easily, see discussion.

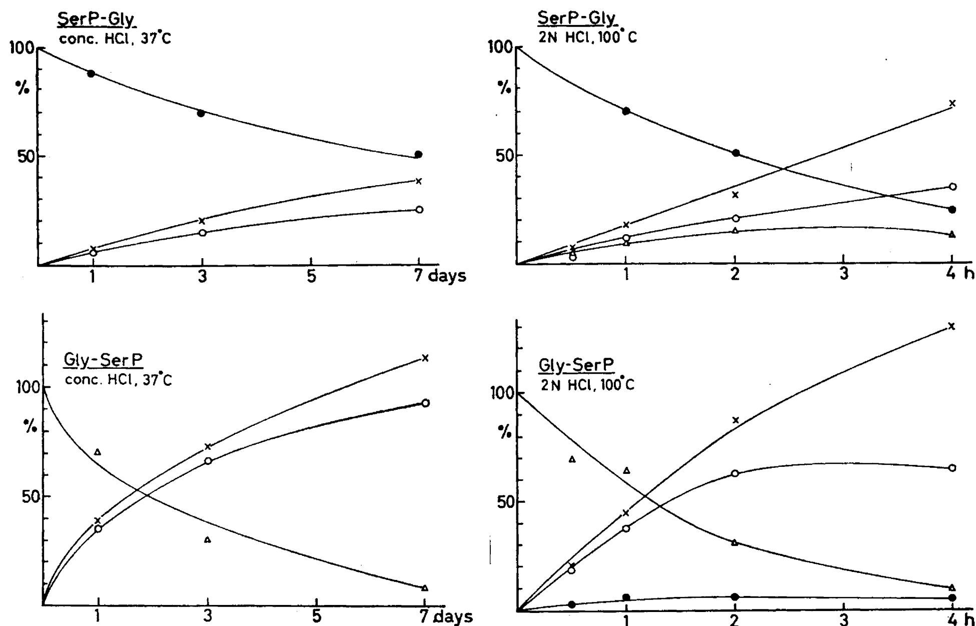


Fig. 2. Rates of hydrolysis of SerP-Gly and Gly-SerP, of formation of dephosphorylated amino acids and peptides and of SerP, and the formation of phosphopeptide with an inversed amino acid sequence. Measured as changes in ninhydrin colour; zero time colour = 100 %.

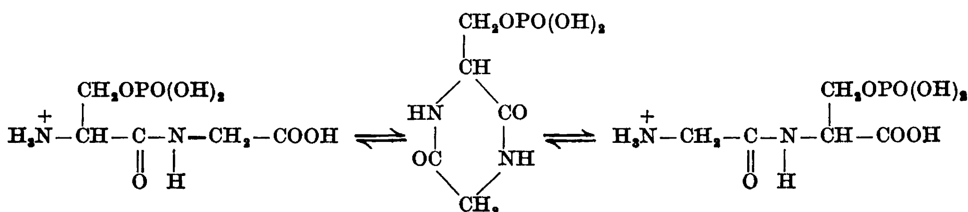
● — ● — ● SerP-Gly
△ — △ — △ Gly-SerP
○ — ○ — ○ SerP
× — × — × Unphosphorylated amino acids and peptides.

In SerP-Gly and Gly-SerP, the hydroxyl groups are esterified with phosphoric acid and, therefore, not available for this reaction. As Gly-SerP is hydrolysed almost exclusively to glycine and SerP, the acyl shift mechanism is not responsible for the higher sensitivity of this phosphopeptide to acid hydrolysis.

Martin has recently discussed the mechanism of the acid hydrolysis of dipeptides in aqueous solution, and the effects of the different side chains of the α-amino acids on the rate of hydrolysis [23]. According to Martin, the mechanism is the same as for amides in general. In the first, fast step, a proton is reversibly added to the peptide bond nitrogen atom, forming an amide cation. In the second, rate-controlling step, a water molecule is added to the carbonyl carbon atom in a bimolecular substitution. A consequence of this mechanism is, that substituents at the β-carbon atom of an N-terminal amino acid have only steric effects on the rate of hydrolysis, while substituents in the C-terminal amino acids produce mainly polar effects. According to this, Gly-SerP would be expected to be hydrolysed at the peptide bond appreciably faster

than SerP-Gly, as in fact was found experimentally. Firstly, the steric hindrance of the side chain of O-phosphorylserine should in the N-terminal position, as in SerP-Gly, keep water molecules from coming in contact with the carbonyl carbon atom of the intermediate amide cation to a much greater extent than the two hydrogen atoms of glycine in Gly-SerP; this effect is shown by the side chain of serine (Ref.[23], Table 3). Secondly, the electronattracting phosphate group of O-phosphorylserine in the C-terminal position as in Gly-SerP should increase the rate of hydrolysis as is found in the case of C-terminal dicarboxylic acid [23].

Schaffer *et al.* have shown [4] that SerP-Gly, during hydrolysis in 2 N HCl, undergoes sequence inversion to Gly-SerP to an appreciable extent. Thompson and Thompson have suggested [3] that this inversion, which is supposed to go *via* the diketopiperazine [1], leads to the more stable peptide sequence as in the conversion of glycylvaline to valylglycine [2]. However, the experimental results obtained here show, that SerP-Gly is at least as stable as Gly-SerP in hydrochloric acid, and that Gly-SerP also undergoes sequence inversion:



If the mechanism for the hydrolysis of dipeptides is also applicable to diketopiperazines, the intermediate above should show preferential ring-opening to give SerP-Gly. As in fact more Gly-SerP is formed by sequence inversion than SerP-Gly, the above diketopiperazine seems to be formed faster from SerP-Gly than from Gly-SerP.

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REFERENCES

1. Sanger, F. *Advances in Protein Chem.* **7** (1952) 1.
2. Sanger, F. and Thompson, E. O. P. *Biochem. et Biophys. Acta* **9** (1952) 225.
3. Thompson, E. O. P. and Thompson, A. R. *Fortschr. Chem. Org. Naturstoffe* **12** (1955) 270.
4. Schaffer, N. K., Harshman, S. and Engle, R. R. *J. Biol. Chem.* **214** (1955) 799.
5. Flavin, M. *J. Biol. Chem.* **210** (1954) 771.
6. de Verdier, C.-H. *Acta Soc. Med. Upsaliensis* **60** (1955) 48.
7. Hipp, N. J., Groves, M. L. and McMeekin, T. L. *J. Am. Chem. Soc.* **79** (1957) 2559.
8. Williams, J. and Sanger, F. *Biochem. et Biophys. Acta* **33** (1959) 294.
9. Schaffer, N. K., Simet, L., Harshman, S., Engle, R. R. and Drisko, R. W. *J. Biol. Chem.* **225** (1957) 197.
10. Anderson, L. and Jollès, G. R. *Arch. Biochem. Biophys.* **70** (1957) 121.
11. Oosterbaan, R. A., Kunst, P., van Rotterdam, J. and Cohen, J. A. *Biochim. e Biophys. Acta* **27** (1958) 549; 556.
12. Dixon, G. H., Kauffman, D. L. and Neurath, H. *J. Biol. Chem.* **233** (1958) 1373.

13. Jansz, H. S., Posthumus, C. H. and Cohen, J. A. *Biochim. et Biophys. Acta* **34** (1959) 387; 396.
14. Hartley, B. S., Naughton, M. A. and Sanger, F. *Biochim. et Biophys. Acta* **34** (1959) 244.
15. Mellander, O., Bennich, H. and Fölsch, G. *Acta Soc. Med. Upsaliensis* **64** (1959) 303.
16. Fölsch, G. *Acta Chem. Scand.* **9** (1955) 1039.
17. Fölsch, G. *Acta Chem. Scand.* **12** (1958) 561.
18. Strid, L. Acta Chem. Scand. **13** (1959) 1787.
19. Moore, S. and Stein, W. H. *J. Biol. Chem.* **211** (1954) 907.
20. Gordon, A. H., Martin, A. J. P. and Synge, R. L. M. *Biochem. J.* **35** (1941) 1369.
21. Desnuelle, P. and Casal, A. *Biochim. et Biophys. Acta* **2** (1948) 64.
22. van Tamelen, E. E. *J. Am. Chem. Soc.* **73** (1951) 5773; *cf.* Konstas, S., Photaki, I. and Zervas, L. *Chem. Ber.* **92** (1959) 1288.
23. Martin, R. J. L. *Austral. J. Chem.* **10** (1957) 256.

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