of 0·006 Å.; the bond angles and the planarity of the rings remain unchanged. As stated, the anisotropy of the thermal movement has not yet been taken into account in my structure refinement.

The corrected C—C distances in dibenzene chromium of 1·45 and 1·36 Å. are significantly different from the distance 1·397 Å. found in free benzene[15]. They correspond more closely to the distances found for pure single and pure double bonds between carbon atoms with sp^2 hybridization, which have average values of 1·46 and 1·34 Å. respectively[16]. This indicates that little or no delocalization of bonding electrons takes place within the ligands of $(C_6H_6)_2Cr$. The cation $(C_6H_6)_2Cr^+$, on the other hand, possesses one electron less than the neutral molecule; electron spin resonance measurements have shown the unpaired electron to be distributed over the whole ion[17]. This, in its turn, may lead to delocalization of the bonding electrons within the rings and hence to six-fold symmetry of the ion. The chemical implications of the above hypotheses will be described elsewhere.

I wish to thank Dr. I. Lindqvist and Mr. R. D. Rosenstein for giving me their X-ray diagrams and intensity estimations, to Dr. D. W. Smits for adapting his refinement programmes for the electronic computer *Zebra* to the case of cubic symmetry, and to Mr. H. Schurer for performing most of the calculations on the *Zebra*.

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BIOCHEMISTRY

Amino-acid Sequence about the Reactive Serine of a Proteolytic Enzyme from *Bacillus subtilis*

AN increasing number of hydrolytic enzymes have been reported to be inhibited by di*iso*propylphosphorofluoridate. Where the resulting di*iso*propylphosphoenzyme has been investigated, the phosphate was isolated in the form of phospho-serine peptides and the amino-acid sequence about the reactive serine has been determined in several cases. Trypsin[1] chymotrypsin[2–4], elastase[5,6] and thrombin[7] have the sequence Gly.Asp.Ser.Gly., while horse liver ali esterase[8] and pseudocholinesterase[9] have Gly.Glu.Ser.-Ala.Gly. The proteolytic enzyme subtilisin was isolated from a strain of *B. subtilis* by Güntelberg and Ottesen[10] and they recorded its inhibition by di*iso*propylphosphorofluoridate, while Matsubara[11] obtained a phospho-peptide from strain *N*[1] but did not report any sequence. The present communication deals with an investigation of the enzyme from Novo Terapeutisk Laboratorium known as 'bacterial trypsin', which is similar to, but distinguishable from, the original subtilisin.

A sample was treated with di*iso*propylphosphorofluoridate labelled with phosphorus-32 at *p*H 7·4 and a partial acid hydrolysate was subjected to ionophoresis on Whatman No. 52 paper at *p*H 3·5 in parallel with similarly treated chymotrypsin and liver ali esterase (sample kindly provided by Dr. E. C. Webb). The radioautograph of the ionogram showed that serine phosphate occurred in all three; but the pattern of phospho-peptides from bacterial protease was entirely different from that obtained from either chymotrypsin[6] or liver ali esterase and therefore there must be a different sequence about the labelled site. Repetition of the hydrolysis of samples of di*iso*propylphospho-enzyme, under the same conditions, gave markedly different proportions of the bands within the pattern. The proportions were also affected by subsequent air oxidation and thioglycollic acid reduction, and some individual bands were found to be interconvertible by oxidation or reduction. This suggested that there was, near the labelled serine, an amino-acid which could undergo reversible air oxidation. It seemed probable that this was methionine, which can be readily oxidized to the sulphoxide[12]. The di*iso*propylphospho-enzyme was therefore oxidized with performic acid to convert any methionine into the stable sulphone, and a simplified, reproducible pattern was obtained on hydrolysis. This pattern, which is also obtained if the previous hydrolysates were oxidized with performic acid prior to ionophoresis, is shown in Fig. 1 together with those from di*iso*propylphospho-chymotrypsin and di*iso*propylphospho-liver ali esterase.

The interrelationships of these oxidized bands (numbered in diagram) were determined by study of their behaviour to partial acid hydrolysis and Edman degradation[6] and their chromatographic and electrophoretic mobilities. Additional information about the N-terminal residue was obtained by treating the

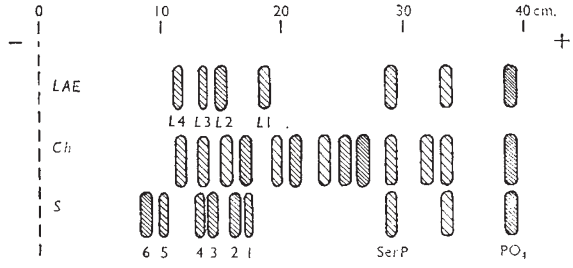

Fig. 1. Diagram of radioautograph of ionogram (*p*H 3·5, 40 V./cm., 2 hr.) of partial acid hydrolysis of di*iso*propylphospho-enzyme derivatives of liver ali esterase (*LAE*), chymotrypsin (*Ch*) and oxidized bacterial protease (*S*). The *LAE* bands are considered to be: *L*1, Glu.SerP.; *L*2, SerP.Ala.; *L*3, Glu.SerP.-Ala; *L*4, Gly.Glu.SerP.Ala. The interpretation of the bands from chymotrypsin is being reported elsewhere (ref. 6)

Table 1

Peptide	Products of partial acid hydrolysis (excluding PO_4 and SerP)	Main radioactive product of Edman degradation	Effect of sodium periodate*
1	1	PO_4	–
2	2	SerP	+
3	1 and 2	1	+
5	1	PO_4	–
6	1, 2, 3 and 5	5	+

* + indicates that the peptide moved faster after treatment; – shows the peptide was not affected.
Peptide 4 is probably a larger peptide the structure of which has not yet been determined.

peptide with sodium periodate solution and observing whether the electrophoretic movement at *p*H 3·5 was affected. N-terminal serine and threonine residues should be oxidized to give a glyoxylic acid residue by loss of ammonia and formaldehyde or acetaldehyde, respectively[13]; so that the radioactive peptide would lose the terminal amino-group and should therefore move faster towards the anode. The results are summarized in Table 1.

A larger sample (50 mgm.) was then treated with di*iso*propylphosphorofluoridate labelled with phosphorus-32, oxidized with performic acid, hydrolysed and the radioactive peptides isolated by paper ionophoresis after removal of the neutral and basic peptides on a column of 'Dowex 50'. The amino-acid composition of each of these, after total hydrolysis, was determined on paper and is presented in Table 2.

Table 2

Peptide	Thr	Ser	Met SO_2	Ala	Asp	Glu	Gly
1	–	× × ×	× ×	–	× ×	*tr*	*tr*
2	× × ×	× × ×	–	–	*tr*	*tr*	*tr*
3	× × ×	× × ×	× × ×	–	×	×	×
5	*tr*	× × ×	× ×	× × ×	*tr*	*tr*	*tr*
6	× × ×	× × ×	× × ×	× × ×	*tr*	–	–

Relative amounts indicated by ×'s and obtained by visual comparison of intensities. *tr* indicates small contamination.

Contamination by non-radioactive, acidic peptides was not great and so from Table 2 and the determined interrelationships of the peptides, represented in Fig. 2, these experiments indicated the sequence Thr.Ser.Met.Ala.

It is interesting that the sequence about the reactive serine in this enzyme is totally different from those determined for other di*iso*propylphosphoenzymes. They had in common an acidic amino-acid, the serine and a small neutral aliphatic residue. Comparing subtilisin with chymotrypsin, trypsin, elastase and thrombin, which are all endo-peptidases with different specificities, it would appear that the sequence immediately about the serine is not responsible for the unusual reactivity towards di*iso*propylphosphorofluoridate which results in the inhibition of the proteolytic activity. Some other grouping, possibly including histidine[14], which is spatially close due to folding of the chain, probably activates the serine.

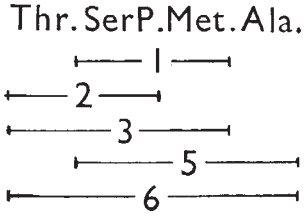

Fig. 2. Interrelationships of peptides. SerP indicates O-phosphoserine

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PHYSIOLOGY

Effect of Testicular Hyaluronidase on the Permeability of Human Arterial Tissue

The action of testicular hyaluronidase on acid mucopolysaccharides isolated from human aortic tissue was established in previous work from this laboratory[1]. The present investigation was undertaken with the purpose of studying the effect of testicular hyaluronidase on the permeability of arterial tissue membranes.

For determination of the tissue permeability, measurements were made of the diffusion coefficients of glucose for the membrane. The coefficient determinations were performed on three samples of the human aorta and three samples of the pulmonary artery, before and after the application of a solution of testicular hyaluronidase to the tissue, using the procedure of Johnson and Kirk[2]. After separation of the adventitia, intimamedia membrane samples of appropriate thickness were prepared. 50 ml. of a solution, consisting of 1 vol. 5 per cent glucose and 4 vol. Krebs's phosphate buffer, *p*H 7·1, were placed in the donor compartment of the diffusion apparatus, and 50 ml. of buffer solution in the recipient compartment. Two 1-hr. diffusion periods were first allowed to establish the permeability of the untreated membrane. Following this, the solutions were removed from the diffusion apparatus and the membrane and compartments rinsed with buffer solution. 5 ml. of a solution of testicular hyaluronidase (Alidase, Searle) in 0·05 *N* acetate buffer, *p*H 6·0 (30 viscosity units/ml.), were then introduced in both sides of the apparatus and were left in contact with the membrane for 2 hr. The membrane and the compartments were again rinsed with buffer solution, and glucose–buffer solution and buffer solution placed in the donor and recipient compartments. Diffusion coefficient measurements were then made in two 1-hr. periods. All experiments were carried out at 37° C. 30–45 min. were allowed to elapse