SUMMARY

1. Glycerol is oxidized and assimilated by washed suspensions of *Mycobacterium smegmatis* and *Mycobacterium butyricum*.

2. The oxidation is completely inhibited by cyanide and partially inhibited by selective agents such as arsenite, iodoacetate and fluoride. Pyruvate accumulates extracellularly in the presence of arsenite only.

3. Study of dialysed extracts has shown that the primary step in the degradation of glycerol is phosphorylation with adenosine triphosphate. Glycerophosphate is subsequently oxidized to triose phosphate and broken down to pyruvate by reactions of the Embden-Meyerhof scheme.

The author is very grateful to Dr N. L. Edson for his advice and continued interest in this investigation.

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The Action of Trypsin on Polylysine

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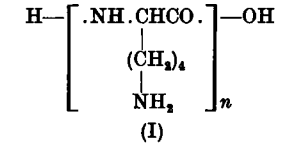
The way in which enzymes act is an outstanding problem in biochemistry, and of all substrates proteins are of predominating interest. Many investigations into the action of proteolytic enzymes have been carried out (Tiselius & Eriksson-Quensel, 1939; Linderstrøm-Lang & Jacobsen, 1941; Haugaard & Roberts, 1942; Winnick, 1944; Christensen, 1949; Desnuelle, Rovery & Bonjour, 1950; Rovery, Desnuelle & Bonjour, 1950; Smith, 1951), but it is a formidable task to elucidate the nature of all the products formed and even more difficult to measure their individual rates of formation. The kinetics of the hydrolysis of simple substrates catalysed by crystalline enzymes (particularly those from the pancreas, Neurath & Schwert, 1950) have been much studied and are relatively well understood. In general, such a substrate contains only one bond capable of being hydrolysed in the presence of the enzyme. Thus the problem of the relative rates of hydrolysis of several susceptible bonds in one substrate molecule does not arise. Synthetic polypeptides containing only one amino acid residue may have many susceptible bonds, but their relatively simple constitution restricts the number of products formed. Even here, the question whether all the susceptible bonds are split at the same rate, or some much faster than others, is not readily answered. This paper describes the attempt to answer this question in one case. The customary methods of following the rate of enzymic hydrolyses by measuring the overall rate of peptide-bond splitting are not directly helpful and the newer

chromatographic techniques have to be employed as well.

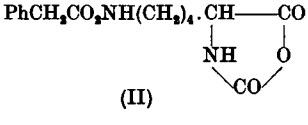
Katchalski (1951) found that polylysine (I) was readily attacked by crystalline trypsin. Estimation of the amino nitrogen (to determine the total extent of hydrolysis) and the carboxyl nitrogen (to determine the amount of amino acid formed) showed that about half the peptide bonds were split and practically no free lysine was formed. We have found by paper chromatography that the main products are dilysine (I; $n=2$) and trilysine (I; $n=3$). L-Lysine was used throughout this work. Apparently peptide bonds adjacent to a carboxyl or amino group are not rapidly split. Thus the nature of the terminal groups in the polymer may be of importance.

METHODS

The polypeptides used in this work were prepared by polymerization of the *N*-carboxyanhydride (II) followed by removal of the benzyloxycarbonyl groups by reduction (Katchalski, Grossfeld & Frankel, 1948). Water was used as initiator so that the polymers possessed terminal carboxyl groups (cf. Stahmann, Graf, Patterson, Walker & Watson, 1951); the products were shown by paper chromatography to be free from lysine and low peptides.



(I)



(II)

In a detailed examination of the kinetics of the action of trypsin on polylysines several of the customary methods for following the hydrolysis of peptides (Neurath & Schwert, 1950) were investigated, but with little success. We then found that the reaction could conveniently be followed by adding alkali to maintain the pH at a constant value.

The relationship between the extent of splitting and the amount of added alkali is obtained as follows: consider the splitting of an isolated peptide bond

$$—CO—NH— + H_2O \rightarrow —CO_2H + —NH_2.$$

Let the ionization constants of the carboxyl and ammonium groups be K_A and K_B. If

$$[—CO_2H] + [—CO_2^-] = [—NH_2] + [NH_3^+] = p,$$

then after the addition of sodium hydroxide the hydrogen-ion concentration will be given by

$$K_A p/(h+K_A) + [OH^-] = ph/(K_B+h) + h + [Na^+].$$

where h is the concentration of H^+. When the pH is near to 7, this becomes

$$[Na^+]/p = K_A/(h+K_A) - h/(h+K_B).$$

Since, in peptides $pK_A \simeq 3$, $K_A \gg [h]$, i.e.

$$[Na^+]/p = K_B/(K_B+h). \quad (1)$$

Consider the hydrolysis of a peptide (whose free α-ammonium group has an ionization constant K_1) to two products. Let the ionization constants of the free α-ammonium groups of these two products be K_2 and K_3, the initial concentration of the original peptide be x and the concentration of the products at any time be p. The amount of alkali required to bring the solution of the original peptide to a hydrogen-ion concentration h (near neutrality) will be $K_1 x/(K_1+h)$. The amount of alkali required to bring both the substrate left and the products formed to the hydrogen-ion concentration h is then

$$K_1(x-p)/(K_1+h) + K_2 p/(K_2+h) + K_3 p/(K_3+h).$$

Thus the amount of alkali required to keep the hydrogen-ion concentration equal to h during the hydrolysis is the difference between these two amounts, i.e.

$$[Na^+] = p[K_2/(K_2+h) + K_3/(K_3+h) - K_1/(K_1+h)]. \quad (2)$$

Thus the amount of alkali taken up is proportional to the number of peptide bonds split. If there is only one susceptible bond, the alkali uptake when all the substrate has been decomposed will give the constant of proportionality. But when there are several susceptible bonds the proportions of the different products formed and their ionization constants have to be known.

The ε-amino groups in lysine derivatives are strongly enough basic to be completely ionized at the pH at which the enzymic reactions were carried out, and so do not affect the amount of alkali taken up during the reaction.

The enzymic hydrolysis of esters has previously been followed by this method (Glick, 1937; Hawes & Alles, 1940; Schwert, Neurath, Kaufman & Snoke, 1948; Schønheyder, 1952), but it has not been used to study the hydrolysis of peptides. It is more convenient than the methods in which samples have to be withdrawn, and is well adapted to the use of small quantities of materials.

The enzymic hydrolysis was also followed by quantitative paper chromatography. Although laborious, this method is particularly valuable when the substrate contains several susceptible bonds because the rates of formation of the individual products can be measured.

EXPERIMENTAL

Materials

Polybenzyloxycarbonyllysines. ε-*N*-Benzyloxycarbonyl-*N*-carboxylysine anhydride (II) was polymerized in water-saturated nitrobenzene at 50°; the polymer was precipitated with light petroleum. The degrees of polymerization were estimated by amino-N (Van Slyke) analyses, and by titration in glacial acetic acid (Hall & Conant, 1927; Seaman & Allen, 1951); the two methods gave concordant results. The abbreviation '-mer' is used for the degree of polymerization, e.g. 230-mer.

Polylysine hydriodides. Reduction of the polybenzyloxycarbonyllysines was carried out with phosphonium iodide in acetic acid; the hydriodides did not move from the original position on paper chromatograms using *n*-butanol:acetic acid:water:pyridine (30:6:24:20, v/v).

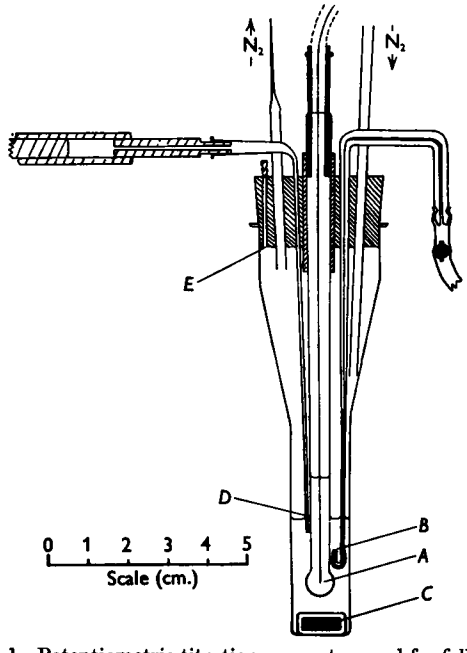


Fig. 1. Potentiometric titration apparatus used for following enzymic hydrolyses. *A*, glass electrode; *B*, calomel electrode; *C*, glass-enclosed iron rod for magnetic stirring; *D*, fine glass tube connected to Agla micrometer syringe; *E*, hole for introduction of enzyme.

Peptides of lysine. The preparation of di-, tri-, tetra- and penta-lysine is described elsewhere (Waley & Watson, 1953). Hexa-, hepta- and octa-lysine were isolated from an acid hydrolysate of polylysine by chromatography on paper. This procedure yielded chromatographically homogeneous samples in sufficient amount for enzymic experiments.

Trypsin. Crystalline trypsin (50% $MgSO_4$) from Armour Laboratories (Charterhouse Street, London, E.C. 1) was used; a stock solution in 0·01 M-$CaCl_2$ kept at 2° retained its full activity for at least a week.

Apparatus

The reaction vessel is shown in Fig. 1. CO_2 was excluded by passing N_2 (previously saturated with water at 25·8°) over the surface of the solution in the vessel, which was in a thermostat at 25·8°. The volume of the solution was 2·75 ml., and was not significantly altered by the alkali added. Initially the solution was brought to pH 7·60; subsequently, the pH dial of the pH meter was left unaltered. The enzyme solution was added, and enough alkali to give a galvanometer deflexion of about one division (a pH change of about 0·04); the time was noted when the deflexion fell to zero, further alkali added and so on.

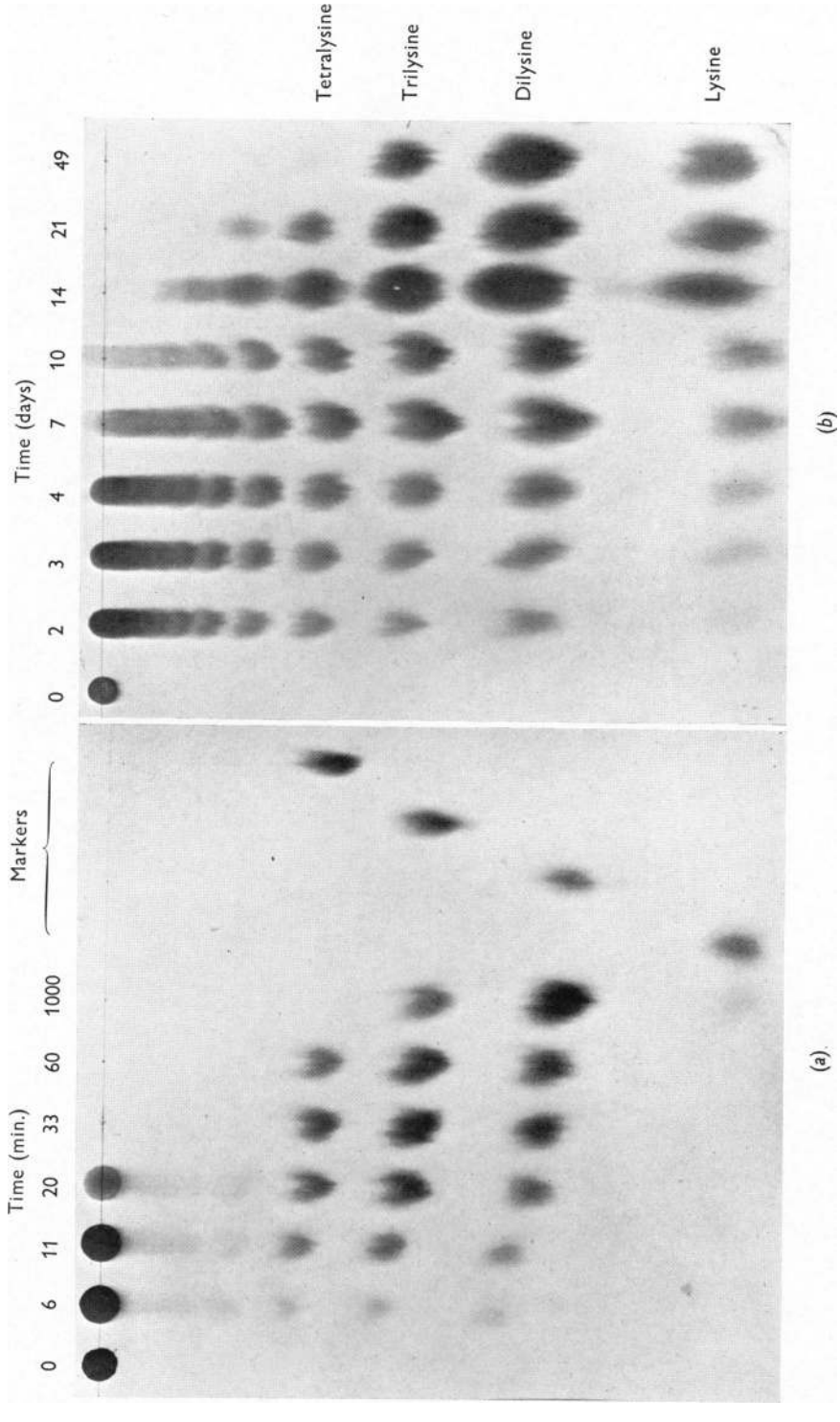
RESULTS

Chromatographic experiments

Qualitative. The nature of the products obtained in the enzyme-catalysed hydrolysis of polymer, with pH maintained at 7·6 by the addition of alkali, is shown in Pl. 3 (*a*) (20·4 mg. polylysine hydriodide (230-mer)/ml.; $4·3 \times 10^{-3}$ mg. trypsin N/ml.; potassium chloride, 0·083 M). The reaction can be divided into two phases: in the first, as the polymer disappeared the major products were di-, tri- and tetra-lysine; in the second phase, as tetralysine disappeared the main products were di- and tri-lysine, together with some lysine (the decomposition of tetralysine has been found to give rise to some lysine). Pl. 3 (*b*) shows the course of an acid-catalysed hydrolysis (20·8 mg. polylysine hydriodide/ml. in constant boiling-point aqueous HBr at 25·8°). Comparable amounts of all the peptides were formed in the early stages, in contrast to the enzymic reaction.

The reactivities towards trypsin of tetra-, penta-, hexa-, hepta- and octa-lysine were compared in the following way. Polylysine hydriodide (330 mg.) in constant boiling-point HBr (15 ml.) was kept at 25·8° for 4 days; the solution was freed from the bulk of HBr by distillation, followed by repeatedly adding water and evaporating. The residue (containing a range of lysine peptides, Pl. 3 (*b*)) was dissolved in water (5 ml.), and 0·5 ml. was fractionated by chromatography on two Whatman 3MM papers, 12 × 8 in. (30·5 × 20·3 cm.), applying a band along the starting line. Synthetic tetralysine was used as a marker. After development with the butanol:acetic acid:pyridine:water solvent for 5 days, the papers were dried at room temperature, heated at 100° for 15 min., and the positions of the bands revealed by their fluorescence in ultraviolet light. The strips of paper containing tetra-, penta-, hexa-, hepta- and octa-lysine were cut out and eluted with 0·1 N-acetic acid, about 0·5 ml. being collected. The solutions were freeze-dried, water added, and the solutions freeze-dried again. The peptides were then dissolved in collidine buffer (pH 7·70) [collidine (Eastman Kodak), 11·6 ml.; 0·2 N-HCl, 140 ml.; water, 250 ml.] containing $8·8 \times 10^{-4}$ mg. trypsin N/ml. The solutions were kept at 25·8°, and at various times samples were transferred to a sheet of Whatman no. 1 paper. Drops of 2 N-acetic acid had previously been placed on the starting line so that the enzymic reaction did not continue on the paper. After development for 60 hr. with the solvent described above the spots were revealed with ninhydrin.

Examination of the intensities showed that while the tetralysine was largely intact after 160 min., all



(*a*) The action of trypsin on polylysine. Substrate concentration: 20·4 mg. polylysine hydriodide/ml.; enzyme concentration: $4{\cdot}3 \times 10^{-3}$ mg. enzyme N/ml. KCl, 0·083M. Temp., 25·8°. (*b*) The action of hydrobromic acid on polylysine. Substrate concentration: 20·8 mg. polylysine hydriodide/ml. in constant boiling-point HBr. Temperature, 25·8°. Solvent: *n*-butanol:acetic acid:water:pyridine (30:6:24:20, v/v).

the higher peptides were about half decomposed after this time. Investigation of the rates of decomposition of tetra- and tri-lysine using higher trypsin concentrations showed that trilysine was about a 100 times less reactive than tetralysine, which was about 10 times less reactive than pentalysine. Dilysine was completely unreactive.

Quantitative. In this experiment the rate of addition of alkali to maintain the pH at 7·6 was measured (thus giving a measure of the overall rate of bond splitting), and samples were withdrawn periodically to measure the rate of formation of the individual products by paper chromatography.

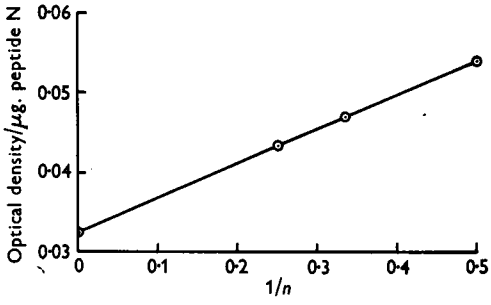
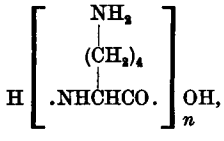


Fig. 2. Colour yield given by peptides of lysine,



in the ninhydrin reaction.

The reaction mixture was 21 mg. polylysine hydriodide/ml.; $2·1 \times 10^{-3}$ mg. trypsin N/ml.; KCl, 0·083 M. The samples (11·3 μl.) were withdrawn and transferred to a sheet of Whatman no. 1 paper; the reaction was stopped with acetic acid as described above. Known amounts of dilysine, trilysine, tetralysine and the polymer were also included on the chromatogram for calibration. After development for 64 hr. with butanol:acetic acid:water:pyridine (30:6:24:20), the colorimetric estimations were carried out as described by Waley & Watson (1953).

There is a linear relationship between the colour yield/mg. peptide N and the reciprocal of the number of lysine residues in the peptide (Fig. 2), from which the colour yield for pentalysine was obtained by interpolation. The results are given in Table 1, and are shown, calculated in molar terms, in Figs. 3 and 4.

The total nitrogen present in 11·3 μl. calculated from a nitrogen estimation was 24·9 μg.; the total found (Table 1) was usually lower than this because neither material between the polymer and pentalysine spots (consisting of hexa- to nona-lysines) nor lysine (formed only from tetralysine), was estimated.

Kinetic results obtained by titration

Enzymic hydrolysis of polylysine. In all the measurements of rates of polymer decomposition the enzyme concentration was 1·6 μg. trypsin N/ml. and except when otherwise stated the runs were conducted in 0·083 M-potassium chloride, to ensure an effectively constant ionic strength.

A typical plot of the alkali uptake is shown in Fig. 5; the reaction is zero order throughout a considerable part of its course. When the initial rate of alkali uptake (v) was plotted against the initial substrate concentration (s), a curve of the form commonly obtained in enzyme-catalysed reactions was obtained, i.e.

$$v = aes/(b+s), \qquad (3)$$

where a and b are constants and e is the concentration of enzyme (Michaelis & Menten, 1913). The parameters a and b were found from the rearranged form:

$$s/v = b/ae + s/ae. \qquad (4)$$

This relationship was obeyed by all of the polymers studied (see Fig. 6). When the substrate concentration was expressed in moles/l. (this point is discussed later), and the enzyme concentration also expressed in moles/l. (taking the molecular weight of trypsin as 36 500), the values of a and b given in Table 2 were obtained.

Table 1. *The rate of enzymic hydrolysis of polylysine followed by quantitative paper chromatography*

(Substrate, 21 mg./ml.; trypsin, $2·1 \times 10^{-3}$ mg. N/ml., in 0·083 M-KCl. Amounts of peptides are expressed in μg. of N present in 11·3 μl.)

Time (sec. × 10^2)	Dilysine	Trilysine	Tetralysine	Pentalysine	Polylysine	Total
3	0·10	0·50	0·72	0·27	24·6	26·2
5	0·50	1·00	0·72	0·74	20·4	23·4
8	1·08	1·92	1·80	0·34	17·7	22·8
11	1·68	3·32	2·80	0·66	14·6	23·1
17	2·75	4·50	3·70	1·32	9·9	22·2
21	3·65	6·30	4·51	1·03	6·2	21·7
30	4·57	8·84	5·04	1·05	2·4	21·9
48	6·36	9·20	5·90	0·01	0	21·5
72	6·55	10·15	5·26	0	0	22·0
182	8·38	9·83	2·83	0	0	21·0

The initial rate of the polymer decomposition was found to decrease when the ionic strength was lowered; thus, for the 70-mer, when the concentration of potassium chloride was reduced by a factor of 4 to 0·021 M, the constants a and b were 13·3 sec.$^{-1}$, and $5{\cdot}8 \times 10^{-4}$ moles/l.

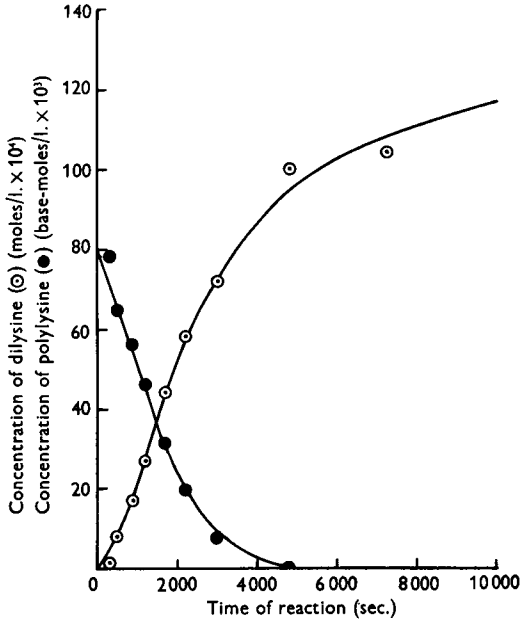


Fig. 3. Formation of dilysine (⊙), and decomposition of polylysine (●), followed by quantitative paper chromatography. Initial polylysine hydriodide, 21 mg./ml., trypsin, $2{\cdot}1 \times 10^{-3}$ mg. enzyme N/ml. KCl, 0·083 M.

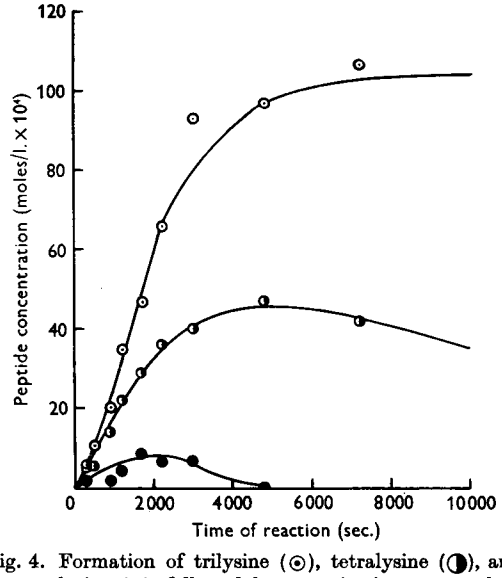


Fig. 4. Formation of trilysine (⊙), tetralysine (◑), and pentalysine (●), followed by quantitative paper chromatography. Conditions as described for Fig. 3.

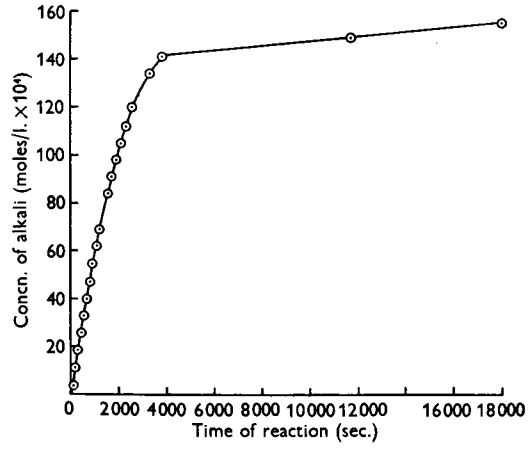


Fig. 5. Amount of alkali added to maintain pH at 7·6 during the enzymic hydrolysis of polylysine. Conditions as described for Fig. 3.

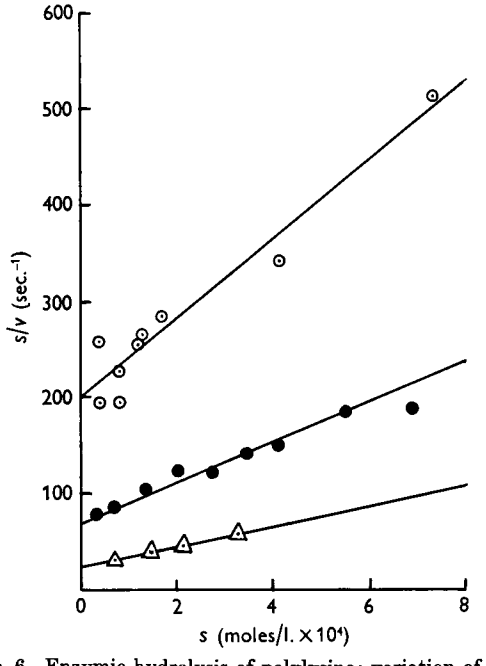


Fig. 6. Enzymic hydrolysis of polylysine; variation of s/v with s, where s is the molar polymer concentration and v is the initial rate of alkali consumption. ⊙, 19-mer; ●, 70-mer; △, 230-mer. Trypsin $1{\cdot}6 \times 10^{-3}$ mg. enzyme N/ml.

Table 2. *Values of the constants in the Michaelis-Menten equation (equation (3)) for polymers of differing degrees of polymerization*

Degree of polymerization of polylysine	a (sec.$^{-1}$)	b (moles/l. × 10⁴)
19	9·4	4·8
70	18·6	3·1
230	36	2·0

Enzymic hydrolysis of tetralysine. Paper chromatography showed that the main product was dilysine, but that some lysine and trilysine were also formed. The kinetics of the hydrolysis were studied by following the uptake of alkali at pH 7·6 in 0·083 M-potassium chloride with 0·024 mg. trypsin N/ml. A straight line resulted when the initial rates were plotted according to equation (4) (Fig. 7); the values of a and b were 0·54 sec.$^{-1}$ and $1{\cdot}75 \times 10^{-3}$ moles/l. respectively.

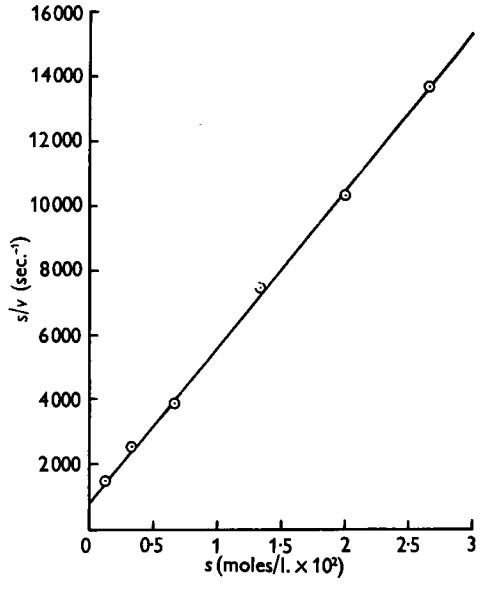


Fig. 7. Enzymic hydrolysis of tetralysine; variation of s/v with s, where s is the molar tetralysine concentration and v is the initial rate of alkali consumption. Trypsin $2{\cdot}4 \times 10^{-3}$ mg. enzyme N/ml.

Enzymic hydrolysis of pentalysine. Paper chromatography showed that the products in this case were di- and tri-lysine. The kinetics were followed as described for tetralysine, but using 0·4 μg. trypsin N/ml. The values of a and b obtained from the linear plot according to equation (4) (Fig. 8) were 12 sec.$^{-1}$ and $5{\cdot}6 \times 10^{-4}$ moles/l. respectively.

DISCUSSION

Some insight into the mode of action of trypsin on polylysine may be obtained from Pl. 3. While the polymer is undergoing decomposition no lysine is formed. Thus the terminal peptide bonds are not attacked. The main question is whether all the bonds, apart from the immune end bonds, are equally susceptible (random fission) or whether some bonds are broken much more readily than others (selective fission). The acid-catalysed hydrolysis of polylysine (Pl. 3) shows two important features: (*a*) lysine is formed; (*b*) comparable amounts of all the peptides are formed early in the course of the reaction. The acid-catalysed hydrolysis appears to be of the random-fission type. The trypsin-catalysed hydrolysis, on the other hand,

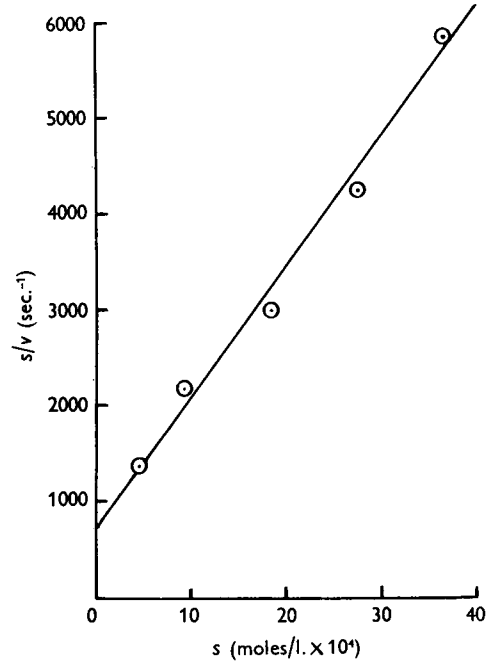


Fig. 8. Enzymic hydrolysis of pentalysine; variation of s/v with s, where s is the molar pentalysine concentration and v is the initial rate of alkali consumption. Trypsin 4×10^{-4} mg. enzyme N/ml.

produces initially mostly di-, tri- and tetra-lysine. This suggests that the bonds most readily split are those near the end of a chain unless the missing higher peptides (penta- to octa-lysine) are particularly reactive. If the velocity constants of decomposition of these peptides were much higher than those of their formation from polymer, their concentrations would always be very low. The chromatographic experiments described above, however, showed that the peptides (penta- to octa-lysine) were decomposed at roughly the same rate by trypsin, and (as is shown in Fig. 9) pentalysine is broken down about as fast as the polymers.

Susceptible bonds

...lysyl—lysyl—lysyl—lysyl—lysyl—lysyl—lysyl—lysyl—lysine

8 7 6 5 4 3 2 1

(III)

In considering in more detail the action of trypsin on the polymer one may start by examining the results obtained by paper chromatography (Table 1 and Figs. 3 and 4). Pl. 3 showed that the reaction could be divided into two phases and this is confirmed by Figs. 3 and 4; in the first phase, lasting about 5000 sec., the polymer is decomposed and di-, tri- and tetra-lysine are rapidly formed, while pentalysine is formed and then decomposes. In the second phase tetralysine slowly decomposes,

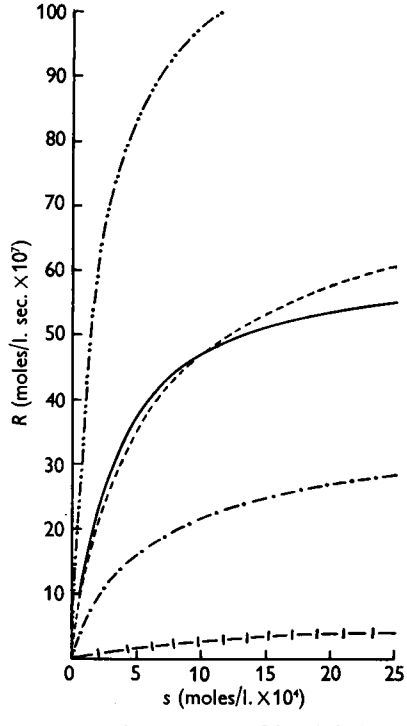


Fig. 9. Variation of initial rate of bond fission (R) with substrate concentration (s). −|−|−|, Tetralysine; - - - - -, pentalysine; −·−·−·−·−, polylysine (19-mer); ——, polylysine (70-mer); −··−··−··−, polylysine (230-mer). Calculated for a trypsin concentration of $1{\cdot}6 \times 10^{-3}$ mg. enzyme N/ml.

mainly to dilysine, the amount of trilysine not changing appreciably. The first phase of this reaction was also followed by the titration method (Fig. 5). From the following argument the contribution of formation of di-, tri-, tetra- and penta-lysine to the total rate of alkali uptake is deduced. When a molecule of a small peptide (e.g. dilysine) is split from the large polymer chain the pK values of the original polymer (e.g. n-mer) and final polymer (n-2 mer) will not differ appreciably, so that the contribution of this step to the total amount of alkali consumed will be governed by the pK of the α-amino group of dilysine, i.e. equation (2) applies with $K_1 = K_3$. The initial rates of formation of the peptides are given in Table 3, together with the values of $K_2/(K_2+h)$ at pH 7·6, using the pK values given previously (Waley & Watson, 1953). Thus the rate of uptake of alkali due to the splitting off of each peptide can be calculated (Table 3, column 4). The initial rate of alkali uptake obtained from Fig. 5 was $6{\cdot}06 \times 10^{-6}$ moles/l. sec., so that the liberation of di-, tri-, tetra- and penta-lysine accounts for more than half this amount. Since the original polymer had over 200 peptide bonds, the main reaction is clearly the formation of the small peptides mentioned above: the question raised above, therefore, is answered in favour of selective fission.

Table 3. *Contribution of the individual steps in the polymer breakdown to the total alkali uptake*

Peptide	Initial rate of formation ($10^6 \times$ moles/l.sec.)	$K_2/(K_2+h)$	Contribution to alkali uptake ($10^6 \times$ moles/l.sec.)
Dilysine	1·2	0·523	0·63
Trilysine	1·7	0·686	1·17
Tetralysine	1·3	0·738	0·96
Pentalysine	0·8	0·772	0·62
	5·0		3·38

It follows that most of the bonds being broken during the enzymic hydrolysis, are those near one or both ends of the polymer chain. It may be assumed that the enzyme must be attached to the polymer in the vicinity of a given bond for this bond to be broken, since trypsin has one 'active centre' per molecule (Balls & Jansen, 1952). Assuming that the enzyme may combine with any residue and that the complex so formed can only break down in one way, one arrives at the following scheme:

$$x_n + E \underset{k_2'}{\overset{k_1'}{\rightleftarrows}} (x_n E)_I \xrightarrow{k_3'} x_{n-2} + x_2 + E \qquad (5)$$

$$x_n + E \underset{k_2''}{\overset{k_1''}{\rightleftarrows}} (x_n E)_{II} \xrightarrow{k_3''} x_{n-3} + x_3 + E, \qquad (6)$$

$$x_n + E \underset{k_2'''}{\overset{k_1'''}{\rightleftarrows}} (x_n E)_{III} \xrightarrow{k_3'''} x_{n-4} + x_4 + E, \qquad (7)$$

where x_n represents the polymer (degree of polymerization n) and E the trypsin. If the total enzyme concentration is E_0, and

$$K_m' = (k_2' + k_3')/k_1', \quad K_m'' = (k_2'' + k_3'')/k_1'', \text{ etc.},$$

then applying the stationary state method the equations have the form

$$\mathrm{d}[x_2]/\mathrm{d}t = k_3' E_0 [x_n]/K_m' \{1 + [x_n]\, \Sigma(1/K_m)\}, \qquad (8)$$

for the initial rates of formation of dilysine, trilysine, etc. The total initial rate of bond splitting (R) is then

$$R=[x_n]\,E_0\,\Sigma(k_3/K_m)/\{1+[x_n]\,\Sigma(1/K_m)\}, \quad (9)$$

where $\Sigma(1/K_m)=1/K'_m+1/K''_m+1/K'''_m+\ldots,$

and $\Sigma(k_3/K_m)=k'_3/K'_m+k''_3/K''_m+k'''_3/K'''_m+\ldots.$

The term 'initial rate' is used in the conventional sense; the initial rate of formation of products is, strictly, zero in any scheme in which the products are formed from a complex. As in other work with enzymes, the complex must have been formed very rapidly, since if the complex were only slowly formed the rate of formation of the products would at first increase and then decrease; in fact this rate decreases steadily. The use of the stationary state method in this connexion has been discussed elsewhere (Waley, 1953).

Equation (9) is of the same form as the empirically found equation (3), i.e. that of the Michaelis-Menten equation with a Michaelis constant of $1/\Sigma(1/K_m)$ and a specific rate for the decomposition of the enzyme-substrate complex of $\Sigma(k_3/K_m)/\Sigma(1/K_m)$. The relative rates of formation of the products are, from equation (8), independent of the initial concentration of the polymer; let it be assumed that they are also independent of its degree of polymerization in order to calculate the values of $\Sigma(1/K_m)$ and $\Sigma(k_3/K_m)$. From the data in Table 3 it can be shown that 0·76 mole of alkali is taken up for each mole of bonds split, so that the rates of alkali uptake (v) can be converted to rates of bond splitting (R), from which the values of $1/\Sigma(1/K_m)$ and $\Sigma(k_3/K_m)/\Sigma(1/K_m)$ given in Table 4 were obtained.

Table 4. *Values of the constants in the Michaelis-Menten equation (equation (9)) for tetra-, penta- and polylysines*

Substrate	$10^4/\Sigma(1/K_m)$ (moles/l.)	$\Sigma(k_3/K_m)/\Sigma(1/K_m)$ (sec.$^{-1}$)
Polylysine (19-mer)	4·8	12
Polylysine (70-mer)	3·1	24·5
Polylysine (230-mer)	2·0	47
Tetralysine	17·5	2·7
Pentalysine	5·6	30

On this scheme of polymer breakdown the relative rates of formation of the products are

$$d[x_2]/dt:d[x_3]/dt:d[x_4]/dt\ldots=k'_3/K'_m:k''_3/K''_m:k'''_3/K'''_m\ldots. \quad (10)$$

It has already been mentioned that the main reaction is the formation of di-, tri-, tetra- and pentalysine, so that the values of k_3/K_m must be lower for splitting in the middle of the chain. There are two extreme cases: either the enzyme combines preferentially with the residues near the end of the chain; or the enzyme combines equally readily with any residue, but only those complexes formed with residues near the end of the chain have specially labile bonds. In the latter case the residues in the middle of the chain would slow the reaction down, so that the overall rate of hydrolysis of the larger polymers would be slower than that of the smaller ones. In fact, this is not the case (Fig. 9). Thus the variation in the values of k_3/K_m referred to above must be caused by variations in k_1 or k_2.

Chromatographic experiments showed that tetralysine decomposed to lysine, dilysine and trilysine. When one mole of tetralysine was decomposed 0·20 mole of alkali was needed to maintain the pH at 7·6; if the overall reaction takes the course

$$3x_4 \rightarrow 4x_2+x_3+x,$$

then, using equation (2), the calculated alkali consumption is 0·19 mole of alkali per mole tetramer decomposed. Since the reaction is composite, the values given in Table 4 have the same significance as they do for the polymers.

When one mole of pentalysine was decomposed, 0·40 mole of alkali was needed to keep the pH at 7·6, and this factor was used to obtain the values of the constants given in Table 4; the value of the factor found from equation (2) was 0·44.

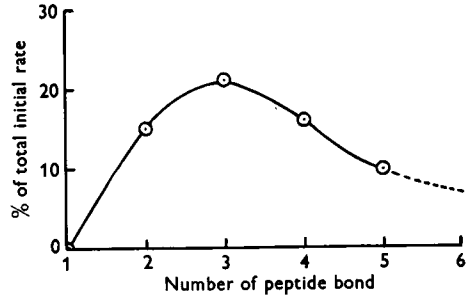


Fig. 10. Variation of reactivity of bonds with position in polymer chain; fission of bond no. 1 gives lysine, no. 2 gives dilysine, etc.

The initial rates of bond fission (for a trypsin concentration of $1·6\times10^{-3}$ mg. enzyme nitrogen/ml.) for all the substrates examined are plotted against the substrate concentrations in Fig. 9. The curves were derived from the straight lines of Figs. 6–8 by conversion of the rates of alkali consumption to rates of bond fission.

Some insight into the reasons for the relative lability of the peptide bonds in polylysine in the trypsin-catalysed hydrolysis can be obtained from the initial rates in Table 3. In Fig. 10 these rates are expressed as percentages of the total initial rate plotted against the position of the peptide bond in the polymer chain. The shape of this curve can be accounted for by supposing that there are two opposing factors governing the relative lability of the bonds: (*a*) the nearer the bond is to the reactive group at the end of a chain, the more slowly does the enzyme attack it; (*b*) the further the bond is from the middle of the chain, the more rapidly does the

enzyme attack it. The existence of the first factor is well known; trypsin does not catalyse the hydrolysis of peptide bonds adjacent to an unmasked amino group (Neurath & Schwert, 1950) and the same appears to hold for carboxyl groups. It is presumably this factor which is responsible for the marked gradation in the susceptibility of the lower peptides of lysine. The existence of the second factor mentioned above is more surprising, and may be connected with a difference in configuration of the residues near the end of a chain. In the form of a film cast from aqueous solution polylysine hydriodide was shown to belong to the class of mainly α-folded polypeptides (A. Elliott, private communication), and it seems reasonable to suppose that the configuration is the same in aqueous solution. Now the end residues of a mainly α-folded polypeptide chain are believed to exist largely in the unfolded, β, configuration (C. H. Bamford & W. E. Hanby, private communication) since the ratio of the amount of β- to the amount of α-form increases with decreasing degree of polymerization (Bamford, Hanby & Happey, 1951). Thus the end residues in polylysine probably exist largely in the β-configuration. If, then, we assume that trypsin combines preferentially with residues in the β-configuration, the second factor referred to above is explained. This may be correlated with the fact that denatured proteins are, in general, much more susceptible to enzymic hydrolysis than native proteins, and furthermore that in some cases native proteins are predominantly in the α- and denatured proteins in the β-configuration (Ambrose & Elliott, 1951).

The rate of hydrolysis of polylysine (70-mer) was found to depend on the ionic strength. Reducing the ionic strength increased the Michaelis constant, i.e. the enzyme has less affinity for the substrate. This is probably connected with the extent to which the polymer chain is coiled, since the viscosities of polylysine were found to depend greatly on the nature of the medium in a way characteristic of poly-electrolytes (Fuoss, 1951).

It is interesting that both random fission and selective fission occur in the enzymic hydrolysis of polysaccharides. Attack of amylose by α-amylase apparently occurs by random fission (Meyer & Gonon, 1951; Roberts & Whelan, 1952) and here also end bonds are immune. The enzyme β-amylase, on the other hand, attacks amylose to give maltose as sole product, so that the fission is selective; also, during the course of the reaction, only amylose and maltose are present (Kerr & Cleveland, 1951) so that this is a particularly clear example of the 'explosion' theory sometimes suggested for the action of enzymes on proteins (Tiselius & Eriksson-Quensel, 1939; Winnick, 1944). The contrast between the modes of action of α- and β-amylase is shown clearly when maltohexaose is the substrate: with α-amylase, the products are three molecules of maltose and one of maltotriose (Roberts & Whelan, 1952): with β-amylase, only maltose is formed (Bailey, Whelan & Peat, 1950).

SUMMARY

1. The action of trypsin on peptides and polymers of lysine has been investigated. Chromatographic methods have been used both to identify the products formed and to follow their rate of formation. The rate of peptide-bond splitting has been estimated by measuring the amount of alkali required to maintain the pH constant during the enzymic hydrolysis.

2. The chief products from polylysine are dilysine and trilysine. The end bonds (next to a carboxyl or amino group) are not split, but those near the end are split preferentially, so that the main reaction initially is a stepwise formation of the lower peptides.

3. Pentalysine breaks down to dilysine and trilysine at a rate comparable with the rate of breakdown of the polymers. Tetralysine reacts more slowly, giving mostly dilysine; trilysine is much less reactive and dilysine is completely inert.

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Studies in Detoxication

54. THE METABOLISM OF BENZENE. (*a*) THE FORMATION OF PHENYLGLUCURONIDE AND PHENYLSULPHURIC ACID FROM [^{14}C]BENZENE. (*b*) THE METABOLISM OF [^{14}C]PHENOL

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Parke & Williams (1953) have shown, using [$^{14}C_1$]benzene, that phenol is a major metabolite of benzene in rabbits. This phenol, however, is not excreted as such but as the glucuronide and ethereal sulphate. Porteous & Williams (1949) have estimated the glucuronic acid and ethereal sulphate outputs of rabbits receiving benzene, but their results represent the conjugates of all the phenolic metabolites of benzene, that is, phenol, quinol, catechol and hydroxyquinol. The use of [^{14}C]-benzene has now enabled us to determine the amount of benzene excreted as phenylglucuronide and phenylsulphuric acid.

In the intact animal, benzene gives rise to *trans-trans*-muconic acid, and it is possible that phenol is an intermediate in the formation of this acid. To test this possibility ^{14}C-labelled phenol, obtained as a metabolite of benzene in our previous experiments (Parke & Williams, 1953), was used. This phenol, since it is obtained from [$^{14}C_1$]benzene, is presumably a mixture of four isomeric [^{14}C]phenols, namely [1-, 2-, 3- and 4-^{14}C]phenols. Using this phenol, it was also possible to find out how much administered phenol was excreted as glucuronide and ethereal sulphate and how much was oxidized to quinol and catechol. ^{14}C-Labelled phenylglucuronide was also prepared from the urine of rabbits receiving labelled phenol or benzene. With this compound the reliability of the usual systematic lead-acetate-precipitation procedure for the isolation of glucuronides from urine could be tested.

EXPERIMENTAL

Measurement of radioactivity. The methods used were essentially the same as described earlier (Parke & Williams, 1953).

Reference compound. In one experiment the diphenylmethyl ester of β-phenyl-D-glucuronide was used. The compound is new and was prepared by treating phenylglucuronide (0·5 g.) dissolved in methanol (5 ml.) with an ethereal solution (10 ml.) of diazodiphenylmethane (0·5 g.) (Elvidge, Linstead, Sims & Orkin, 1950). After standing overnight the crystals which separated were collected and recrystallized from ethanol (yield, 0·6 g.). The *diphenylmethyl ester* formed colourless needles, m.p. 172°, $[\alpha]_D^{20} - 72 \pm 1°$ in ethanol (*c*, 1). (Found: C, 68·8; H, 5·8. $C_{25}H_{24}O_7$ requires C, 68·8; H, 5·5%.) (Light absorption in ethanol; $\lambda_{max.}$ 207, 212, 214, 261–262, 267, 274 mμ. with $\epsilon_{max.} \times 10^{-3}$ 17·1, 18·3, 17·1, 1·27, 1·62, 1·08, respectively.)

Experiments with [^{14}C]*benzene*

Phenylglucuronide. Rabbits were fed with [$^{14}C_1$]benzene (cf. Parke & Williams, 1953) and the urine collected for 48 hr. To one-quarter of the urine was added 200 mg. of β-phenyl-D-glucuronide dihydrate (m.p. 162°, cf. Garton, Robinson & Williams, 1949) dissolved in a little water. The glucuronide was then isolated from the urine as the basic lead salt as described by Porteous & Williams (1949). After removal of the lead with H_2S and concentrating, the glucuronide was obtained as a pale-yellow gum. This was purified by dissolving in methanol, filtering and evaporating to dryness *in vacuo*. The residue was recrystallized twice from water and its specific activity determined. The glucuronide (m.p. 161°) was converted into the triacetyl methyl ester (m.p. 115°) and then to phenylglucuronid-