REVERSIBLE INACTIVATION OF LYSOZYME DUE TO N,O-PEPTIDYL SHIFT

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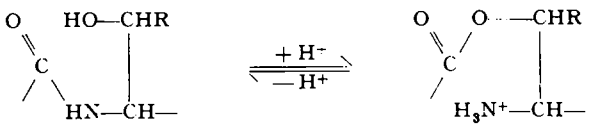
In recent years, the N,O-acyl migration at the hydroxyamino acid residues of proteins (N,O-peptidyl shift) has attracted much interest.



N-peptidyl form O-peptidyl form

This reaction, originally proposed as a hypothesis by BERGMANN, BRAND AND WEINMANN[1], is now well established by the accumulated evidence. Thus, DESNUELLE AND CASAL[2] and DESNUELLE AND BONJOUR[3] were able to produce evidence that the acid hydrolysis of the peptide bonds adjacent to the hydroxyamino acid residues proceeded via O-peptidyl intermediates. ELLIOTT[4,5], in careful studies on silk fibroin and lysozyme, conclusively demonstrated the transformation of these proteins into their O-peptidyl forms under the influence of concentrated sulfuric acid. Moreover, it was to be expected that an N,O-acyl migration would be reversible, and this reversion was realized by keeping the O-peptidyl forms in weakly alkaline solution. It is noteworthy, that the products of the reversion differed in properties from the starting products, and this was ascribed to cross linking at the tryptophan residues induced by the strong acid. Later, MCCONNELL *et al.*[6,7] confirmed and extended these observations to gluten and gliadin. The report by CHIBNALL AND REES[8], that the N,O-peptidyl shift took place during the acid catalyzed esterification of insulin, was of special interest for the present study, since the conditions were here less drastic than in the aforementioned studies.

The aim of the present investigation[9,10] has been to study the relationship between the N,O-peptidyl shift and the enzymic activity, in the hope of shedding some new light on the mechanism of the latter. For this task, full advantage of the reversibility of the reaction ought to be taken, and therefore an attempt was made to find such experimental conditions that concomitant, irreversible changes of the enzyme protein were avoided. Lysozyme was chosen because of its known stability towards denaturation. It was then found that anhydrous formic acid at room temperature induced an extensive N,O-peptidyl shift in this enzyme, and that a complete reversal to an apparently native enzyme could be brought about in aqueous solution at pH 7.5–8.5. These findings form the basis of the present study.

References p. 623.

EXPERIMENTAL

Methods

The total nitrogen was determined according to the micro-Kjeldahl procedure with mercuric oxide as a catalyst.

The amino nitrogen determinations were made according to Van Slyke with the modifications introduced by KENDRICK AND HANKE[11]. The reaction time was 15 min, unless otherwise stated.

The lysozyme activity was assayed by the turbidimetric procedure of SMOLELIS AND HARTSELL[12] with the following modifications. The buffer was 0.05 *M* potassium biphthalate, pH 5.4. The concentration of *Micrococcus lysodeikticus* was kept at 0.9 mg per ml and the temperature at 25° C. The lysis was followed turbidimetrically in a Coleman Junior spectrophotometer, model 6A, set at 570 mμ.

The autotitrations at constant pH were made in a pH-stat of similar construction to the one described by JACOBSEN AND LÉONIS[13], but not provided with a recorder. The standard alkali, 0.05 *N* sodium hydroxide, was added from an Agla micro burette. Protection from atmospheric carbon dioxide was ensured by an overpressure in the titration vessel of nitrogen gas washed in a solution of sodium hydroxide.

Melting points were determined on a Fisher-John melting block.

Materials

Lysozyme hydrochloride was prepared from egg white according to ALDERTON AND FEVOLD[14]. The preparation was crystallized five times and then dried *in vacuo* over phosphorous pentoxide at 110° C to constant weight. When subjected to ion exchange chromatography on IRC-50 (XE 97), according to TALLAN AND STEIN[15], the preparation showed only one peak.

Analyses. N, 16.9; amino-N, 0.67.

*O-Acetyl-*DL*-serine* was prepared according to SAKAMI AND TOENNIES[16].

*N-Carbobenzoxy-*DL*-seryl-*L*-leucine benzyl ester* was prepared by the mixed anhydride procedure[17]. N-Carbobenzoxy-DL-serine[18] (2.4 g) was dissolved in dioxane (12 ml), tri-*n*-butylamine (2.4 ml) added and the solution cooled in ice-water. Under vigorous stirring ethyl chlorocarbonate (1 ml) was added dropwise and the stirring continued afterwards for 10 min. Then, L-leucine benzyl ester hydrochloride[19] (2.6 g) and tri-*n*-butylamine (2.4 ml) in dioxane (12 ml) were added, and the reaction mixture left overnight at room temperature. On the addition of water, a yellowish oil separated which was taken up in chloroform (20 ml). The chloroform layer was washed in succession with dilute solutions of hydrochloric acid, sodium bicarbonate and with water and finally dried over anhydrous sodium sulfate. After concentration, addition of petroleum ether and cooling, crystallization set in. Recrystallization from chloroform-petroleum ether. M.p. 107° C. Yield, 2.4 g.

Analysis. Found: N, 6.3. $C_{24}H_{30}O_6N_2$ (442.5) requires N, 6.3.

DL*-Seryl-*L*-leucine*. N-Carbobenzoxy-DL-seryl-L-leucine benzyl ester (1.0 g) was hydrogenated with palladium as the catalyst in the usual manner. After concentration of the reaction mixture to a few ml and addition of ethanol and ethyl ether, crystallization set in. Recrystallization from water-acetone. Yield, 0.47 g.

Analysis. Found: N, 12.7. $C_9H_{18}O_4N_2$ (218.3) requires N, 12.7.

*O-Acetyl-*DL*-seryl-*L*-leucine*. DL-Seryl-L-leucine (0.218 g) was dissolved in anhydrous acetic acid (2 ml) made 0.6 *M* with respect to perchloric acid. Acetic anhydride (0.52 ml) was added slowly and with cooling. After 1 hour at room temperature, water (0.04 ml) was added and the reaction mixture was left for another hour, then cooled to 0° C and tri-*n*-butylamine (0.16 ml) added. Crystallization was brought about by the addition of several vol. of acetone. Yield, 0.160 g.

Analyses. Found: N, 10.7; amino-N (reaction time, 4 min), 5.2. $C_{11}H_{20}O_5N_2$ (260.3) requires N, 10.8; amino-N, 5.4.

Formic acid (Merck, 98–100%) was made anhydrous through prolonged drying over boric anhydride as described by SCHLESINGER AND MARTIN[20]. The desired volume was distilled off *in vacuo* with protection against moisture in all glass still immediately before use.

Ethyl ether was checked for absence of peroxides prior to use.

RESULTS

A. Inactivation of lysozyme in formic acid

Dried lysozyme hydrochloride was dissolved in anhydrous, freshly distilled formic

References p. 623.

acid (10 mg per ml) and the solution kept in a closed vessel at 25° C*,**. At suitable intervals 1 ml samples in duplicate were withdrawn and immediately precipitated at —10° C with 10 vol. of ethyl ether. The precipitate was centrifuged off in the cold, washed with ethyl ether and dried. One of the identically treated samples was assayed directly, and the other after reactivation (*cf.* Section B). The dry product was dissolved without difficulty in 5.0 ml of distilled water (pH approx. 3). After 30 min, suitable dilutions for activity measurement were made with 0.05 *M* potassium biphthalate buffer, pH 5.4.

The enzymic activity dropped slowly and was completely lost after approx. 16 hours (Fig. 1)***.

B. Reactivation

The other sample in the duplicate was dissolved in 3.0 ml of water and cautiously brought to pH 7.5–8.0 (glass electrode) with 0.1 *N* sodium hydroxide and kept at this pH for 4 hours with occasional adjustments of pH. The solution was subsequently brought to pH 5 with 0.1 *N* hydrochloric acid and diluted with water to 5.0 ml. After 30 min suitable dilutions for activity measurement were made as described above.

The treatment in weakly alkaline solution restored the enzymic activity of lysozyme at any stage of inactivation (Fig. 1).

C. Alkali consumption during reactivation

It was observed that the restoration of activity was accompanied by a drop in the pH of the solution. Parallel measurements of reactivation and alkali consumption were therefore made.

The measurements were made with the aid of the autotitrator which was first

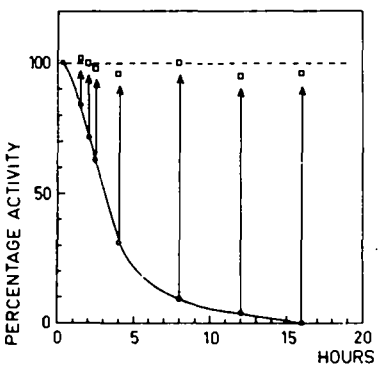


Fig. 1. Inactivation of lysozyme in formic acid (–○–○–) and reactivation at pH 7.5 (–□–□–). The ordinate represents the percentage activity of the native enzyme.

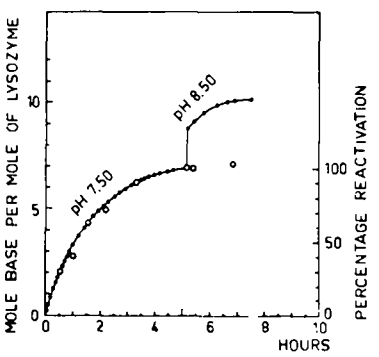


Fig. 2. Alkali consumption (–●–●–) and reactivation (○) of inactivated lysozyme at 20° C. Reactivation presented in percentage of total reactivation during measurement.

* Some of the earlier experiments were carried out at 20° C.

** The formic acid solution remained colorless during the entire experimental period, but when allowed to stand for several days, a purple color began to appear, indicating a certain extent of destruction in the tryptophan residues.

*** The low pH of 5.4 for the assay was chosen for convenience, since here reactivation did not complicate the measurements. However, occasional measurements at higher pH's (up to pH 8.8) have confirmed the results obtained at the lower pH. To minimize reactivation during the assay at higher pH's, a rapid assay was devised requiring 2.5 min and a correspondingly higher concentration of enzyme.

References p. 623.

set to pH 7.5 and, after cessation of the alkali consumption, to pH 8.5. Samples for activity measurements were withdrawn at regular intervals. The course of one experiment is presented in Fig. 2. A sample of lysozyme, which had been inactivated in formic acid for 8 hours at 20° C, was titrated at the concentration of 15 mg per ml of water. The initial activity was 7% of full activity and during the titration up to pH 7.5 the activity increased to 17%*.

The reactivation followed closely the alkali consumption at pH 7.5. Analysis of the figures revealed that both reactions obeyed first order kinetics with a specific rate constant of $1.15 \cdot 10^{-2}$ min^{-1} at 20° C. At pH 8.5 an additional base consumption was observed here as well as in other titrations of lysozyme, but this was not accompanied by a further reactivation. No base consumption was observed after raising the pH to 9.5.

The base consumption at pH 7.5 and the corresponding first order rate constant were also determined for lysozyme that had been exposed to formic acid for varying lengths of time (Fig. 3 and Table I). The consumption increased steadily at first and finally reached a constant value of 11 equivalents per mole of lysozyme after approximately 15 hours.

Titrations were also made at pH 6.2 and 8.4. At the lower pH the reaction was still clearly first order (Table I). At the higher pH the reaction was much faster, but deviated markedly from the first order course.

TABLE I

SPECIFIC RATE CONSTANTS OF THE ALKALI-CONSUMING REACTIONS

Temperature, 20° C.

Preparation	*Duration of treatment in formic acid (hours)*	*pH of measurement*	*First order specific rate constant* (min^{-1})
1. Inactivated lysozyme	2	7.5	$1.12 \pm 0.10 \cdot 10^{-2}$
2. Inactivated lysozyme	4	7.5	$1.25 \pm 0.10 \cdot 10^{-2}$
3. Inactivated lysozyme	8	7.5	$1.20 \pm 0.20 \cdot 10^{-2}$
4. Inactivated lysozyme	12	7.5	$1.05 \pm 0.10 \cdot 10^{-2}$
5. Inactivated lysozyme	16	7.5	$1.05 \pm 0.10 \cdot 10^{-2}$
6. Inactivated lysozyme	20	7.5	$1.05 \pm 0.05 \cdot 10^{-2}$
7. Inactivated lysozyme digested with pepsin	20	7.5	$4.95 \pm 0.10 \cdot 10^{-3}$
8. Inactivated lysozyme	20	6.2	$1.30 \pm 0.10 \cdot 10^{-3}$
9. O-Acetyl-DL-seryl-L-leucine	—	6.2	$5.7 \pm 0.2 \cdot 10^{-4}$
10. O-Acetyl-DL-seryl-L-leucine	—	6.5	$1.3 \pm 0.2 \cdot 10^{-3}$
11. O-Acetyl-DL-seryl-L-leucine		6.8	$1.8 \pm 0.1 \cdot 10^{-3}$
12. O-Acetyl-DL-serine	—	7.5	$3.1 \pm 0.2 \cdot 10^{-3}$

In one experiment the inactive lysozyme was digested with pepsin prior to the titration. To a solution of 100 mg of inactive lysozyme in 10 ml of water was added 2 mg of crystalline swine pepsin. The digestion was allowed to proceed for 20 hours at 25° C and pH 3, and was followed by amino nitrogen determinations. At zero time the amino nitrogen was 0.96% and after 20 hours had reached the value of 1.66%. This increase corresponded to the cleavage of approx. 8 peptide bonds per mole of lysozyme. Two ml of the digest was diluted to 3 ml with water and titrated at pH 7.5 in the usual manner. A first order reaction, although somewhat slower than for the

* Owing to insufficient stirring during the initial, rapid addition of alkali.

References p. 623.

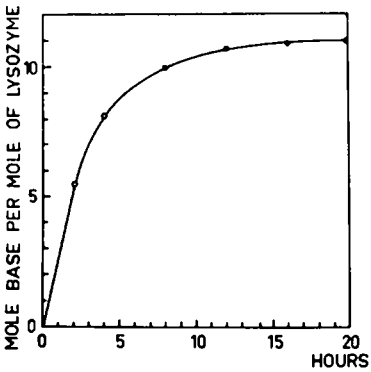


Fig. 3. Total alkali consumption at pH 7.5 of lysozyme treated for varying lengths of time in formic acid at 25° C.

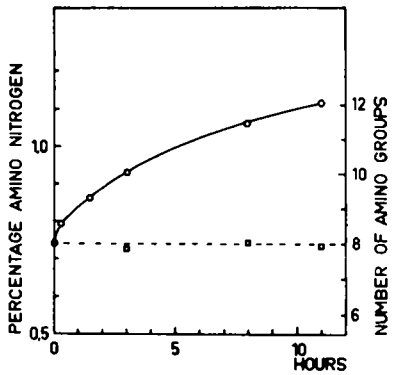


Fig. 4. Amino nitrogen during treatment of lysozyme with formic acid (-O-O-), and following storage at pH 7.5–8.0 (-□-□-).

comparable, undigested sample, was observed (Table I). The alkali consumed, 10.7 equivalents per mole of lysozyme, was practically the same as for the undigested sample.

D. Amino nitrogen during inactivation and reactivation

The experimental conditions were those described in Sections A and B.

During the inactivation a progressive increase in amino nitrogen was observed (Fig. 4)*. After storage in weakly alkaline solution, the amino nitrogen of the preparation returned to the figure for the native lysozyme.

E. Reactions of model substances

The O,N-acyl shifts in O-acetylserine and O-acetylserylleucine were followed by autotitration at fixed pH under conditions similar to those described for the inactivated lysozyme (*cf.* Section C).

An aqueous solution of O-acetylserine ($2.4 \cdot 10^{-3} M$) was titrated at pH 7.5 and O-acetylserylleucine ($5.0 \cdot 10^{-3} M$) at pH 6.2, 6.5 and 6.8.

The conversion into the corresponding N-acetyl derivatives was demonstrated by the absence of free amino nitrogen in the resulting preparations. Also, for acetylserylleucine the conversion was followed by subjecting samples of the reaction mixture to paper chromatography in *n*-butanol–acetic acid–water, where a definite difference in R_F-values between the O- and N-acetyl dipeptides was observable.

The reactions were in all instances first order (Table I). It should be noted that, in contrast to the findings with inactivated lysozyme, no additional base consumption was observed after raising the pH to 8.5.

The effect of formic acid on the constituent amino acids of lysozyme was also studied.

The amino acids were treated in groups (Table II) made up of approximately equimolar amounts of the constituent amino acids. Each mixture was dissolved separately in anhydrous formic acid and the solutions kept for 7.5 hours at 20° C.

* It should be noted that these determinations were not satisfactory from the quantitative point of view, since the values obtained for the inactivated enzyme were appreciably dependent on the length of the reaction time in the Van Slyke apparatus. The probable explanation is that for steric reasons some of the amino groups are poorly accessible to the reagent.

References p. 623.

TABLE II

ALKALI CONSUMPTION OF AMINO ACIDS TREATED WITH ANHYDROUS FORMIC ACID

Group of amino acids	*Alkali consumption per mole of amino acids (equivalents)*	
	pH 7.5	*pH 8.5*
I. Glycine, alanine, valine, isoleucine, leucine, proline	0.000	0.006
II. Tyrosine, histidine	0.012	0.041
III. Serine, phenylalanine, tryptophan, lysine	0.007	0.006
IV. Threonine, aspartic acid, glutamic acid, arginine	0.003	0.000
V. Cystine, methionine	0.007	0.155

The formic acid was subsequently evaporated *in vacuo* over pellets of sodium hydroxide and the dry residues were dissolved in water. The alkali consumption at pH 7.5 and 8.5 was measured using the autotitrator as described in Section C.

Even if the base consumption were arbitrarily ascribed to any one of the amino acids in one group, it would at pH 7.5 not exceed 0.03 equivalents per mole of amino acid. At pH 8.5 only cystine-methionine showed a significant base consumption, which, under the same assumption, would correspond at most to 0.34 equivalents per mole of amino acid. The cause of this reaction was not further investigated.

F. Electrophoretic measurements

The electrophoretic mobilities of the native, inactivated and reactivated forms of the enzyme were determined.

The measurements were made in a Tiselius-Svensson electrophoresis apparatus. In order to prevent reactivation during the experiments, a low pH was chosen for the measurements. An acetate buffer, pH 3.90 and ionic strength 0.1, was used throughout. The inactivated preparation was obtained by keeping the enzyme in formic acid for 20 hours (*cf.* Section A). The reactivated form was prepared by titration at pH 7.5 and afterwards at pH 8.5 (Section C). In one instance the latter titration was omitted. All solutions were dialyzed with stirring against the buffer for 12 hours at 4° C prior to electrophoresis. The protein concentration was 1–2%.

The results are presented in Fig. 5 and Table III. Evidently, the inactivated form had a higher cathodic mobility than both the native and the reactivated enzyme,

TABLE III

ELECTROPHORETIC MOBILITIES OF NATIVE, INACTIVATED AND REACTIVATED FORMS OF LYSOZYME

Acetate buffer, pH 3.90 and ionic strength 0.1.

Lysozyme preparation	*Electrophoretic mobility* $\times 10^{4} \times cm^{2} \times volt^{-1} \times sec^{-1}$
Native	− 0.84
Inactivated	+ 1.01
Reactivated (to pH 8.5)	+ 0.84

References p. 623.

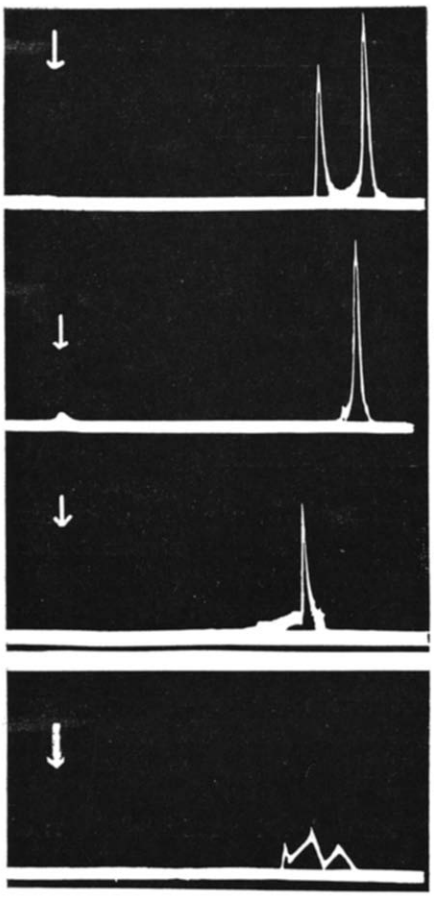


Fig. 5. Electrophoretic patterns of (A) mixture of native and inactivated lysozyme, 160 min, (B) inactivated lysozyme, 150 min, (C) lysozyme reactivated to pH 8.5, 150 min, and (D) lysozyme reactivated to pH 7.5, 150 min. Acetate buffer, pH 3.90 and ionic strength 0.1. Ascending limbs. Arrows indicate starting points.

and, moreover, the latter two had identical mobilities. A striking electrophoretic homogeneity was observed for all preparations excepting the one reactivated only at pH 7.5. The latter preparation displayed peaks of intermediate mobilities. However, also here the reduced cathodic mobility was apparent.

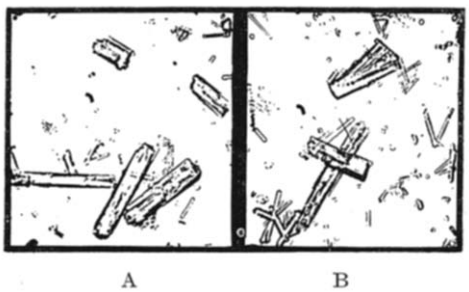


Fig. 6. Crystals of (A) native lysozyme, and (B) reactivated lysozyme.

G. Crystallization of reactivated lysozyme

The reactivation was carried out as described in Section C with the modification that the concentration of protein was held at 2%. The solution was then concentrated to 4% and the preparation crystallized as described for lysozyme hydrochloride[14].

The appearance of the crystals did not differ from that of native lysozyme hydrochloride (Fig. 6)*.

H. N-Terminal amino acids of native and reactivated lysozyme

These determinations were made by the PTC (phenylthiocarbamyl) method essentially as described earlier[21, 22, 23].

In agreement with an earlier finding[24], lysine was found to be the sole N-terminal amino acid of native lysozyme, and the reactivated enzyme was found not to differ in this respect**.

* In addition to the bipyramidal crystals of lysozyme hydrochloride described by ALDERTON AND FEVOLD[14], we have regularly obtained the thin, rectangular plates depicted in Fig. 6.

** Attempts to demonstrate in the inactivated enzyme the presence of serine and threonine with free amino groups using FDNB (Sanger's reagent) have failed. In these experiments the coupling with FDNB was made at pH 5 for 8 hours, and the expected mono- and di-DNP-

References p. 623.

I. Substrate affinity of inactivated lysozyme

The recent report[25] of a substrate competition between inactive methyl lysozyme and native lysozyme prompted a study of a possible relationship of similar kind in our case.

Mixtures of native and inactivated lysozyme in varying proportions were assayed by the usual procedure (Table IV).

No competition for the substrate between the formic acid-inactivated and the native enzyme was noticeable.

TABLE IV

ASSAYS OF NATIVE AND INACTIVATED LYSOZYME, SEPARATELY AND IN MIXTURES

Native lysozyme μg per ml	*Inactivated lysozyme μg per ml*	ϵ_{570} *after 15 min*
0	0	0.66
1.25	0	0.49
2.50	0	0.40
3.75	0	0.33
0	25	0.66
0	50	0.66
0	100	0.66
1.25	50	0.50
2.50	50	0.40
3.75	50	0.34
2.50	12.5	0.41
2.50	25	0.41
2.50	100	0.42

DISCUSSION

If the chemical changes in the enzyme protein are first considered, the experimental evidence clearly supports the contention of a reversible N,O-peptidyl shift. Thus, the treatment with formic acid under nonhydrolytic conditions causes, in accordance with expectations, an increase in the amino nitrogen as well as in the net positive charge of the protein, as demonstrated in the electrophoretic measurements. Moreover, these changes are completely reversed under conditions where an O,N-peptidyl shift is known to occur, *i.e.* in aqueous solution near neutrality. Additional support is offered by the concomitant release of hydrogen ions that this mechanism requires, on the reasonable assumption that at pH 7.5 the amino groups involved are at least partly in their ammonium form. Also, the kinetics of the reaction agree with those found for the O,N-acyl migration in synthetic model substances. A postulated extensive formylation of susceptible amino acid side chains in the protein would hardly account for all of the observed facts, and is not supported by the result of the attempt to formylate the free amino acids under the same experimental conditions (*cf.* Section E).

The base consumption at pH 8.5 may also be caused by an O,N-peptidyl shift,

derivatives of lysine were obtained. With the same goal in mind, we also tried to demonstrate a deficit in the content of serine and threonine following deamination with nitrous acid. Although a deficit was regularly found, the figures varied erratically and are, therefore, not presented here. Similar difficulties have been encountered by earlier workers[3,5].

References p. 623.

which for some reason occurs less readily. The incompleteness of the shift at pH 7.5 is indicated by the electrophoretic pattern of the resulting enzyme preparation (Fig. 5).

The complete restitution of enzymic activity, crystallizability, electrophoretic mobility and amino nitrogen rules out the possibility of an *extensive* denaturation of the protein in formic acid, though a more limited derangement of the structure is still possible. The observations of HARRINGTON[26] on the appearance of acidic and basic groups, due to rupturing of polar, intramolecular hydrogen bonds during *denaturation*, might have a bearing on our findings, if a *renaturation* were postulated to take place during the reactivation. However, it is difficult to conceive of any such pair of polar groups, which on renaturation of the protein would give rise to the observed release of hydrogen ions at pH 7.5. The rejection of this interpretation is also supported by the observation that the base consumption of the inactivated enzyme at pH 7.5 is quantitatively unaffected by a prior pepsin digestion, which should have effectively prevented a renaturation.

The close connection of the reactivation with the base-consuming reaction, *i.e.* the N,O-peptidyl shift, is established by the identical kinetics of the reactions. A competitive effect of the inactivated form on the substrate could not be demonstrated in contrast to what has earlier been found for methyl lysozyme.

The exact number of peptide bonds undergoing the N,O-peptidyl shift is difficult to establish. The potential figure for lysozyme should obviously equal the sum of the number of hydroxyamino acid residues, *i.e.* 10 serine and 7 threonine[27,28]. The figure obtained from the amino nitrogen determinations should be disregarded in this connection for reasons already given (*cf.* footnote, Section D). On the other hand, the base consumption at pH 7.5, corresponding to 11 groups, is a minimum figure as the pK's of the amino groups are not known. Also, the figure rises somewhat if the base consumption at pH 8.5 is taken into account. Still more difficult to assess is the number of shifts directly involved in the inactivation–reactivation process. It is quite possible that only a fraction of the shifts observed at pH 7.5 is required for complete restoration of the enzymic activity, but the identical figures obtained for the average specific rate constants of the various O,N-peptidyl shifts and for the reactivation process do not permit any conclusions in this respect.

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SUMMARY

It has been shown that lysozyme loses its enzymic activity when stored in anhydrous formic acid solution at room temperature, and that the activity is completely restored in aqueous solution at pH 7.5. The close dependence of the reversible inactivation on a likewise reversible N,O-acyl migration at the hydroxyamino acid residues of the enzyme protein has been demonstrated.

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ENZYMIC CLEAVAGE OF FOLIC ACID BY EXTRACTS FROM HUMAN BLOOD CELLS

I. PREPARATION AND CO-FACTOR REQUIREMENTS OF THE ENZYME SYSTEM

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Several reports have described the inactivation of folic acid encountered during the assay of this vitamin in animal tissues[1]. SILVERMAN *et al.*[2] have demonstrated that folic acid on incubation with rat liver extracts suffers a loss in the growth-promoting properties for *Streptococcus faecalis* R. Comparatively less is known, however, about the enzymes or the pathways through which folic acid is inactivated in animal tissues. During the course of this work two abstracts have appeared which report that rat liver slices[3] and pigeon liver extracts[4] can degrade folic acid with the liberation of a diazo-

References p. 634.