CCXVII. ON MONOLAYERS OF PROTEOLYTIC ENZYMES AND PROTEINS.

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PART I.

THE REACTION KINETICS.

IN a recent communication [Hughes and Rideal, 1933] it was shown that the progress of reactions taking place at an air-liquid interface could be followed by observation of the rate of change of the phase boundary potential. In this paper an account is given of a preliminary investigation of the action of a typical proteolytic enzyme system, trypsin on monolayers of egg-albumin and of caseinogen. This investigation was undertaken because it appeared probable that this type of reaction at a phase boundary frequently occurs in living systems.

The structure of albumin monolayers.

There is a considerable amount of information which indicates that the structure of proteins in bulk solution is different from that which exists when the protein is extended as a monolayer in a film.

The measurements of Svedberg and Nichols [1926] lead us to regard egg-albumin as an isodisperse protein of molecular weight 34,500, and from the constancy of its "molecular" weight it is assumed to be approximately spherical in form [cf. Sørensen, 1915]. The spherical character of the egg-albumin molecule is further supported by an optical examination due to Ettisch *et al.* [1931] who found that egg-albumin solutions in flow exhibit no birefringence and light scattered in the Tyndal beam is polarised. These views are opposed to those of Staudinger who regards eucolloids as existing in the filamentous or distended state in solution. The ease with which the protein particle breaks down outside the region of its stability limits, p_H 4 to p_H 9, suggests that the particle may not be really isodisperse within the limits but may consist of three units each of true molecular weight 11,500, built up into a single net-like unit (the choice of three similar units is based upon the approximate analysis by Vickery and Shore [1932]). A net-like structure has indeed been proposed by Kuhn [1932] for gelatin from an analysis of the viscosities of dilute solutions of this protein. It is clear that whichever view of the structure of egg-albumin in solution be correct, the balance of evidence is against the rod-like or filamentous structure. It has been shown [Hughes *et al.*, 1932; Fosbinder and Lessig, 1933] that a number of proteins may be spread directly as homogeneous surface monolayers from the solid material. Protein monolayers are characterised by the fact that under suitable conditions they form highly elastic gel-like films. For the polydisperse proteins such as gliadin and caseinogen in which the polypeptide chains are probably highly branched the gel state is not acquired until relatively high two-

dimensional pressures (15 dynes/cm.) are applied to the film; this phenomenon is accentuated in films of polypeptide mixtures such as peptone where solid films are formed only under very high compression. In the case of the isodisperse proteins like albumin the characteristic gel-like appearance is quite noticeable at relatively low pressures (< 1 dyne per cm.) and even below these pressures the films cannot be regarded as anything except weak gels developing into more homogeneous gels. If egg-albumin be spread on buffer solution it is found that the rate of spreading and the quantity per cm.² attained at equilibrium suddenly increase several 100 % when the acidity of the substrate is extended beyond the stability region of the isodisperse state; it may be significant that this change coincides with the region of peptic digestion of the film.

Monolayers of the isodisperse and polydisperse proteins are also differentiated by the fact that in the polydisperse proteins such as gliadin and caseinogen, possessing many branched chains, the phase boundary potentials are relatively high whilst for the monodisperse proteins such as albumin they are much lower. The examination of the effect of changing the p_H of the substrate on the phase boundary potential likewise indicates that this is governed by the number and nature of the polar groups in the side-chains and is scarcely affected by the less polar amide linkages in the main polypeptide chain.

In the solid state Astbury and Wood's analysis [1932] has shown that the proteins exist in two characteristic forms—the zig-zag type of extended polypeptide chain, the β -keratin structure, and a chain folded in a series of hexagons of periodicity $5.1 \text{ \AA.}$, the α -keratin or diketopiperazine ring type. Whilst in the surface soluble proteins spreading out into monolayers the chains must be of the original β -keratin type, it seems certain that conversion into the α -keratin or diketopiperazine ring structure takes place in the surface and very rapidly, and it is to this latter structure that the stability of the films is due. Whilst the measurements of the limiting surface concentrations at the stage when gel formation commences are not sufficiently well defined experimentally to confirm this view as to their structure, yet the values obtained for the isodisperse proteins where the branch chains are not so prominent certainly favour this hypothesis. The following experimental observations may be cited in support of this view. The decrease in solubility of natural proteins after being converted into a film is well known and has been examined in detail by many investigators, notably Ramsden. Monolayers of egg-albumin may be heated to 85° and quickly cooled without apparent alteration in their physical properties. Natural albumin is readily dispersed by caustic soda more concentrated than $p_H 11$ but monolayers of albumin are very resistant even to $N/10$ caustic soda. On allowing even dilute solutions of proteins to stand [*cf.* du Noüy, 1926] the micelle in solution comes up to the surface and there spreads, undergoing the process of denaturation and loss of solubility. Finally, albumin in the film state is readily attacked by enzymes, a property not associated with natural albumin in bulk which appears to be very resistant, and this ease of attack is associated with the presence of diketopiperazine and anhydride rings in the molecule [Matsui, 1933]. It might be suggested that the process of denaturation at interfaces is associated with or may even consist in the conversion of the β -keratin or zig-zag structure into the α -keratin or diketopiperazine ring form.

We have noted that the examination of the phase boundary potential of a number of protein monolayers suggested that the main contribution to the phase boundary potential was from the polar groups in the side-chains and that the $-\text{CO}-\text{NH}-$ linkage of the main chain contributed but little. On hydrolysis, if only one or more of these links be broken to form shorter polypeptide

chains terminating in $-\text{COOH}$ and $-\text{NH}_2$ respectively, the phase boundary potential should increase until the peptide chain becomes so small that dissolution commences, and only then will the phase boundary potential commence to fall. As an alternative method, hydrolysis consists in the breaking either of an amide linkage near the end of a chain or two amide linkages close together in a chain and the small fragment containing relatively many polar groups thus released goes into solution. Under these conditions there will be a gradual fall in the phase boundary potential as the protein passes through the stages of peptone, polypeptide, tri- and di-peptide and simple amino-acid disintegration. As will be noted, this latter change is the one found experimentally and it is indeed possible to express, in a qualitative manner at least, the stages of disintegration of the protein by observation of the magnitude of the phase boundary potential.

Since the concentration necessary to form films is of the order of 10^{-7} g. per cm^2 , the method of surface potentials is not only particularly well suited for the characterisation and control of purity of biological materials which possess large surface potentials and are available only in small quantities but also in its sensitivity in measurement of chemical reactivity and rates of reaction for substances in almost sub-analytical quantities. In the case of these enzyme reactions in particular the kinetics can be followed over a convenient period of some twenty minutes at room temperature, and, furthermore, some light is shed on the actual nature of the enzymes themselves.

Thanks to the investigations of Willstätter and Grassman, Waldschmidt-Leitz and his co-workers and Northrop¹, we can distinguish in commercial trypsin solutions digesting egg-albumin at p_{H} 8–9, a proteinase, trypsin, effecting a primary hydrolysis into polypeptides; further hydrolysis proceeds by attack at the terminal groups in the polypeptide chain by polypeptidases. We may note that both the amino- and carboxy-polypeptidases which may be present in trypsin hydrolyse the amide linkages close to a terminal $-\text{NH}_2$ or $-\text{COOH}$ group respectively with the result that small soluble fragments, *e.g.* leucine or tyrosine, are removed from the ends of the polypeptide chains, a point, as we have noted, of some importance in interpreting the effect of hydrolysis on the phase boundary potential. Whether a small soluble fragment is liberated in the action of the primary proteinase, trypsin, on the original protein has not been established but, as we shall note, such a suggestion is supported by the observed change in phase boundary potential. Still further hydrolysis is effected by the dipeptidases present.

EXPERIMENTAL PROCEDURE.

The experimental method employed followed closely that described in previous communications [Schulman and Rideal, 1931; Schulman and Hughes, 1932], with the exception that the apparatus was kept at 25° , the egg-albumin being spread from the solid from a micro-balance on the surface of $M/20$ buffer solutions made up according to the directions of Clark ("Determination of hydrogen ions") over a range of p_{H} 2–12; the solution was contained in a quartz trough of 300 cc. capacity. It was found necessary to take precautions to eliminate the formation of diffusion potentials in the liquid junctions connecting the buffer solution in the trough with the auxiliary electrode and membranes on the electrode itself.

The protein film so spread could be compressed by means of glass barriers to

¹ Compare *Ergebnisse der Enzymforschung*, Band 1, Leipzig, 1932.

the stage where the phase boundary potential on gelation no longer varied appreciably on further contraction; the film in this state possesses a characteristic surface potential dependent only on the p_H of the substrate. The water surface behind the barrier which had been moved to compress the film was left clean. 20 cc. of a suitable solution of the enzyme having stood at room temperature for half an hour in the same buffer solution as that on which the protein film was spread, but $M/10$ in strength (strong buffers being found necessary owing to the acidity of the trypsin), were injected and well mixed from the free side of the barrier into the underlying solution. With the aid of the moving polonium electrode the process of subsequent digestion of the protein film as well as the rate of adsorption of the ferment at the clean surface could be followed.

Owing to the high adsorbability of albumin at the surface of aqueous solutions of albumin, one part in half a million parts forming solid gel films at an air-liquid interface, it was found essential to eliminate soluble proteins from the enzyme. If the soluble protein is not eliminated it forms partially digested protein films at the surface which obscure and inhibit the main reaction so that the reaction appears to proceed in steps or jumps, the dimensions of which are dependent on the time the enzyme has been in solution before being injected into the trough. It was found that on standing at room temperature for half an hour the minute quantities remaining from a Merck's pancreatin were removed presumably by autodigestion. Solutions of pancreatin prepared in this manner were found to possess definite and reproducible phase boundary potentials, which could be employed as a criterion for their freedom from protein. All experiments were carried out with Merck's pancreatin, which gave more uniform results than other varieties.

The change in phase boundary potential due to an albumin film in the gel state on the buffer solution at p_H 7.7 is $\Delta V = 230$ mv. On injection of the pancreatin solution a steady decrease in the phase boundary potential is observed and the film loses its rigidity when the surface potential attains a value of *ca.* $\Delta V = 180$ mv., finally becoming quite fluid. The phase boundary potential finally attains a constant value of $\Delta V = 118$ mv. This value $\Delta V = 118$ mv. is identical with the characteristic value shown by a solution of pancreatin at this p_H so it appears that the protein film has been completely digested. On the protein-free side of the barrier within two minutes of injection of the trypsin solution a potential change of $\Delta V = 118$ mv. is obtained; the capillary active constituents in the pancreatin thus form a vaporous film of characteristic surface potential whose value does not change even on relatively rapid compression or expansion of the surface, a behaviour similar to that of substances which are capillary active and on solution in water form a Gibbs layer which may be regarded as a two-dimensional vapour or gas.

During the reaction the original protein gradually disappears from the film and we may assume that the removal of fragments of the protein from the surface is reflected in the change of phase boundary potential; this assumption is borne out by the fact that identical velocities of reaction are obtained when, instead of measuring the rate of change of phase boundary potential with time, maintaining the area of film constant, the rate of contraction of area of film necessary to maintain a constant phase boundary potential is measured. Experimentally, however, this latter procedure is difficult to carry out, especially in the later stages of digestion.

In Fig. 1 are shown the effects of variations in the concentration of pancreatin and of temperature on the rate of digestion of an albumin film at p_H 7.7.

Fig. 1.

Fig. 2.

Fig. 2 a.

It will be noted from Figs. 2 and 2*a* that the reaction in presence of pancreatin, originally unimolecular, becomes converted on decreasing the concentration of pancreatin and at lower temperatures firstly into an inhibited reaction obeying the Arrhenius expression $\frac{dx}{dt} = k \frac{a-x}{x}$ (Fig. 2), then the rate of displacement of the by-products of the digestion by the trypsin alone governs the reaction velocity and the reaction follows Schutz's law $\frac{dx}{dt} = \frac{k}{x}$ (Fig. 2*a*). Curves *a*, *b*, *c* and *d* are given as examples for the two expressions, *c* showing irregularity owing to diffusion to the surface of a weak trypsin solution.

A comparison of the actual velocity constants obtained for the digestion of films of caseinogen with the approximate values obtained for the reaction in bulk phase under similar conditions indicates that they are of the same order of magnitude, the chief difference is that the transition from a unimolecular surface reaction velocity to an inhibited reaction following Schutz's law takes place as a result of a smaller change in the concentration of the pancreatin; this is doubtless due to the greater facility with which by-products from the reaction can diffuse away from the disperse micelle in the bulk phase and the decreasing ability of the trypsin in the lower concentration to displace the by-products formed initially on the surface, since on complete digestion the pancreatin ultimately displaces all the by-products of digestion, as indicated by the attainment of identical values of ΔV_∞ on both sides of the barrier.

In Fig. 3 is plotted the logarithm of the change in phase boundary potential with the time and in Fig. 4 the unimolecular velocity constants respectively as a function of the p_H over the range of activity of the pancreatin. It will be noted that the surface reaction follows very closely that of the bulk reaction revealing a marked optimum between p_H 8 and p_H 8.4.

In the neighbourhood of the optimum the reaction only reveals a unimolecular reaction constant over the first few minutes, by-products being formed so rapidly that inhibition of the reaction sets in after a short period; thus the reaction as a whole proceeds more rapidly at p_H 7.5 than at p_H 8.2.

It is somewhat remarkable that the reaction at p_H 7.5, in spite of the complexity of the process, appears to be uniformly unimolecular over the whole course of the reaction, suggesting that the slowest of the enzyme reactions is that involving the first breakdown by the proteinase. Another noticeable feature of these reactions in films is that occasionally (approximately one experiment in every twenty) a period of induction was noted before the reaction commenced with its normal velocity, the usual phenomenon met with in the tryptic digestion of albumin when studied in bulk phase.

The specific proteolytic enzymes.

In order to examine the mechanism of tryptic hydrolysis in more detail, a number of experiments were carried out with proteinase and carboxypolypeptidase. We are indebted to Prof. J. H. Northrop of the Rockefeller Institute at Princeton for a sample of proteinase or crystalline trypsin (N) prepared by ammonium sulphate precipitation from pancreatin and also to D. R. P. Murray of the Sir William Dunn Biochemical Laboratory at Cambridge for a sample of proteinase (M) and of carboxypolypeptidase (P) prepared according to the method of Waldschmidt-Leitz by preferential adsorption on alumina.

In contrast with solutions of pancreatin, which at p_H 7.7 form only vaporous films with very low phase boundary potentials, these specific proteolytic ferments form solid gel-like films, possessing characteristics very similar to films of egg-

Fig. 3.

Fig. 4.

albumin, the phase boundary potential of the proteinases being $\Delta V = 220$ mv. and of the carboxypolypeptidases $\Delta V = 200$ mv. The proteinase N formed solid films very rapidly after injection of the solution into the buffer solution in the trough but some ten and twenty minutes respectively elapsed before the proteinase M and the carboxypolypeptidase P yielded solid films.

Solutions of these proteases were injected at p_H 7.7 under films both of egg-albumin and of caseinogen. In the examination of the stages of proteolytic digestion in films, caseinogen being polydisperse possesses certain advantages over egg-albumin in that not only is the change in phase boundary potential greater than for albumin in the region of gelation, but, accompanying a high surface pressure the region of gelation is smaller and more sensitive to changes in pressure.

In Figs. 5 and 6 are given a summary of the experimental results of the action of these enzymes on the two proteins.

Fig. 5.

Fig. 6.

It will be observed that the proteinases N and M reduce the surface potential to a definite value, M, to a slightly greater extent than N, a fact probably due to the difficulty in elimination of the last traces of polypeptidases by the adsorption

process. Owing to the fact that only small quantities were available it has been impossible to determine the reaction constants, but, with the exception of the digestion of egg-albumin by Northrop's proteinase which follows a zero order, they appear to be unimolecular. In one case the carboxypolypeptidase P was injected into the solution of proteinase which had already digested a film of egg-albumin to the first stage. This resulted in a further stage of digestion with a concomitant fall in the surface potential of a further 20 mv. Likewise a mixture of M and P enzymes digested a caseinogen film with a change of 55 mv. compared with 25 mv. with the proteinase alone.

On comparison of these values with those obtained in the digestion of either egg-albumin or of caseinogen by pancreatin containing the complete series of enzymes, it is found that in the proteolytic digestion by the proteinase N about 10 % of the original protein has left the surface in some soluble form, whilst a further 25 % leaves the surface through the addition of the carboxypolypeptidase P.

On the other hand, about 15 % and a further 16 % leave the surface in the action of the ferments N and P respectively in the case of caseinogen. Another interesting feature observed in the digestion of a film of caseinogen, which possesses a region of gelation very sensitive to pressure (the rigidity of the gel being imperceptible when the phase boundary potential falls below 260 mv.), is that it is still in a gelatinous state when digested by the proteinase N, but on digestion by the ferment M which contained a slight trace of a carboxypolypeptidase a small further digestion reflected by a change of only 4 mv. in the phase boundary potential caused it to assume a fluid condition. The proteinase, however, rising to the free surface of the water on the other side of the glass barrier, forms a solid film. These conditions on each side could be maintained for several hours. The proteinase which itself forms a solid film does not displace the liquid film of the partly digested caseinogen owing to the higher surface pressure of the latter, and continuous gel-like films of proteins do not appear to be formed except at an air-liquid interface, no formation taking place underneath a monolayer.

PART II.

SOME STRUCTURAL CHARACTERISTICS OF THE ENZYMES.

The phase boundary potential of trypsin.

In Fig. 7 are plotted the phase boundary potentials of an albumin film in the gel state with that of a solution of trypsin for comparison as a function of the p_H of the medium.

It is interesting to note that at p_H 4 trypsin causes a marked alteration in the phase boundary potential, viz. $\Delta V = 320$ mv.; no tryptic activity is noted here, the film is gel-like and possesses all the properties of an albumin protein film. As we pass into the region of proteolytic activity the phase boundary potential falls, the surface loses its gel-like character and becomes perfectly fluid and similar to that of a vaporous or gaseous two-dimensional phase. Pepsin exhibits a similar behaviour, forming an albumin-like film at p_H 7 but a fairly mobile surface with a low phase boundary potential at p_H 2, the peptic optimum. The experimental evidence suggests that the trypsin solutions, even when prepared with the precautions stated, still contain small quantities of protein which form films at p_H 4; but at p_H 8 the rate of arrival of the protein micelle from the interior with

the subsequent spreading is equalled by the rate of tryptic digestion of the protein. In the region p_H 8 to 12 the phase boundary potentials are very similar as well as the fluidities of the surface, but at p_H 12 no surface tryptic activity is noted. It is probable that the traces of protein left in the solution of the enzyme in the micellar form are completely dispersed at this p_H and thus cannot form surface films by spreading from micelles. With strong solutions of trypsin or on

Fig. 7.

the addition of extra albumin solid gel-like films are eventually formed. Albumin when spread from a solid on to the surface forms a perfectly stable gel-like film at this p_H . It is clear that the characteristic surface potential which remains almost constant over this range of p_H , whilst no criterion for the activity of the ferment, is an indication that a distinct change has taken place in respect to the protein constituents in the surface solution.

That the films formed by pancreatin on acid substrates really consist of protein-like material was confirmed by the fact that they are readily digested by pepsin injected into the solution at p_H 2 in the usual manner.

The resultant active surface of pancreatin at the optimum p_H evidently contains no protein, although at p_H 2 undigested pancreatin forms films with all the characteristics of protein.

Similar digestion experiments were carried out with pepsin which forms protein-like films on alkaline solutions (p_H 7.7) of slightly lower $\Delta V = 220$ mv. compared with 240 mv. for albumin. These could be digested readily by pan-

creatin. Likewise the protein-like films formed by the proteinases obtained from Prof. Northrop and Dr Murray (see Part I) as well as by the carboxypolypeptidases could be digested in a similar manner by further injection of pancreatin.

Since the protein-like proteinase and the more disperse protein-like carboxypolypeptidase are further digested by addition of the lower trypsin, it would suggest that the various tryptic ferments are associated with their own breakdown product of original protein when in solution at the p_H for optimum activity, and over the inactive region of p_H with proteins albumin-like in character.

It is clear that all these enzyme preparations are associated with the presence of protein-like material which is capable of being digested by other enzymes or by autodigestion and, also, after removal of the protein by digestion it appears that the surface enzymic activity of the solutions remains unimpaired.

The conclusions inferred from these experiments could logically be extended to enzyme solutions which have been digested by other enzymes, *e.g.* pancreatin by pepsin. These have already been examined by Northrop who showed that the enzymic activity in solution decreased as the digestion of the proteinase proceeded. We have found by digesting pancreatin with pepsin at p_H 2 and then bringing the solution back to p_H 7.7 that in agreement with Northrop's observation the tryptic activity in bulk disappears as the peptic digestion becomes more complete. On the other hand the very interesting observation was made that whereas these solutions were inactive for the digestion of caseinogen, albumin or gelatin in the bulk phase, if films were formed of either of the former proteins on these apparently inert solutions, digestion took place rapidly with unimpaired activity.

The method consisted in dissolving 0.3 g. pancreatin and 0.2 g. pepsin in 50 cc. *N*/100 HCl, leaving to digest for varying lengths of time at 38° and injecting the mixture into the trough containing 250 cc. phosphate buffer at p_H 7.4. The changes in films of egg-albumin or caseinogen spread upon the surface were then observed. It was noticeable that on prolonged digestion of the pancreatin by pepsin the surface proteolytic activity became greater until it reached the normal activity of undigested pancreatin after 24 hours' treatment. This was probably due to the removal from the solution by peptic digestion of protein which inhibits the surface reaction. Even after 48 hours' digestion by pepsin a considerable surface tryptic activity was observed. A control experiment indicated that the tryptic activity of pancreatin appeared to be unimpaired when it was dissolved in 50 cc. *N*/100 HCl and the solution subsequently injected into a phosphate buffer at p_H 7.4.

In the curves in Fig. 8 are shown the results obtained and it is seen that this activity for surface digestion is maintained for relatively long periods of time; whilst after only 5 hours' digestion of pancreatin by pepsin the activity in the bulk phase is scarcely measurable when tested with caseinogen and precipitation by acetic acid. Pepsin, on the other hand, is quite inactive on protein films at p_H 7.7 and when the acidity is brought back to p_H 2 it is still completely inactive; this is analogous to its behaviour in the bulk phase.

From the curves in Fig. 1 (Part I) it is seen that the surface activity is very sensitive to changes in the bulk concentration of pancreatin, and we must conclude that in these experiments the concentration of active enzymes in the bulk phase has been left unaltered by the peptic digestion, although the solutions exhibit no tryptic activity in the bulk phase.

Two points emerge from these film experiments; firstly, that the active enzyme can be removed from its protein-like carrier by suitable digestion. This appears to be in agreement with the experiment of Dyckerhoff and Tewes [1933],

who effected an exchange of protein carrier for the pepsin, making edestin and other protein carriers peptically active by precipitation from pepsin solutions, whilst Frenkel [1932] effected a separation of a toxin from its protein carrier, leaving an active solution containing no protein in the case of diphtheria toxin.

Fig. 8. Digestion of albumin films by trypsin solutions which have previously been digested by pepsin at p_H 2. 0.2 g. pepsin + 0.3 g. trypsin in 50 cc. HCl; in bulk, no reaction after 5 hours.

The second point of interest is that solutions of these enzymes from which their normal protein-like carriers have been removed by digestion are incapable of exerting any enzymic activity on proteins except when these latter are presented to them in the particular state or orientation associated with the film. We have so far been unsuccessful in effecting a regeneration of bulk enzyme activity by the addition of a suitable support material, although we have included both inactive enterokinase and edestin, as well as albumin denatured in various ways, including that denatured by film formation, and also substances such as alumina and quartz. The experimental investigation is being continued in this direction.

It has long been known that destruction of enzymic activity in respect to its activity in a bulk phase proceeds rapidly on boiling. A series of experiments was carried out with a view to investigating the existence (in boiled solutions) of any residual enzyme activity to protein monolayers. Pancreatin solutions containing 0.3 g. in 20 cc. of buffer solution were boiled for various periods of time and cooled rapidly to 0° and then injected into the trough containing the buffer solutions and their surface enzymic activity measured by the rate of hydrolysis of either albumin or caseinogen films; comparative runs with un-boiled solutions were carried out at the same time. The results obtained are shown in Figs. 9 and 10.

Fig. 9. Digestion of egg-albumin by boiled trypsin solutions p_H 7.8. *a*. Normal 0.1% trypsin solution. *b*. Solution heated for 5 minutes. *c*. Solution heated for 30 minutes. *d*. Solution just brought to boiling.

Fig. 10. Digestion of caseinogen by boiled trypsin solutions p_H 8.2. *a*. Normal. *b*. Heated to boiling at p_H 8.2. *c*. Boiled for 30 minutes at p_H 8.2. *d*. Boiled for 30 minutes at p_H 4.6 and filtered, no reaction.

It will be observed that solutions of trypsin when boiled at p_H 8 for a period even as long as 30 minutes still exert a marked tryptic activity on protein films, the activity being still about one-third that of the original solution, whilst no activity could be noticed in bulk as tested by caseinogen and acetic acid precipitation. It may be remarked that egg-albumin films appear to be more resistant to the boiled enzyme than films of caseinogen, which liquefy and disappear in the normal manner. The ultimate phase boundary potentials obtained are some 40 mv. higher for the boiled than for the unboiled solutions, showing that some change has occurred in the component parts of the pancreatin.

The results of these experiments carried out at p_H 8 are to be contrasted with the results obtained by boiling a trypsin solution at p_H 4.6, filtering off the coagulated protein and bringing the p_H back to 8 again. These solutions exhibit no tryptic activity either in bulk or for protein films and indeed form solid protein-like films themselves at an air-liquid interface which, however, in no way affect the solidity of a gelatinous caseinogen film if a solution of the boiled enzyme be injected underneath. It appears that the enzyme surface activity is in direct relation to the degree of coagulation and consequent insolubility of the protein carrier; for at p_H 8 there is only a slight precipitation of coagulated protein and a lengthening of the digestion period of a film from some fifteen to forty-five minutes.

It might be concluded from these experiments that the enzyme consists of at least two components, a "free enzyme" non-protein in character and a protein possessing a specific structure, generally adsorbing or reversibly binding the free enzyme. The "free enzyme" readily reacts with proteins when presented to it in the form of an orientated protein monolayer but does not react with proteins in solution. To impart activity for a bulk phase reaction to the "free ferment" the latter must be adsorbed on a protein possessing a specific structure, for although it is readily adsorbable by coagulated proteins it is inactive both for surface and bulk reaction in this state. It is possible that the function of active enterokinase is to present such a surface.

It may be supposed that an enzyme consists of a system which includes several separate components, the "free enzyme," which in the case of the proteolytic enzymes appears to be non-protein in character, the free enzyme carrier which is specific and protein-like in character and behaves in a manner similar to that of an orientated monolayer and of surface activators such as enterokinase. The activity of the complex of free enzyme and its carrier is dependent on its environment both in respect to its power of adsorption and its chemical reactivity. It is hoped that further experiments on the relationship between bulk and surface reactivity will throw further light on the characteristics of the enzyme system.

It is interesting to note here that when these proteolytic ferments are treated chemically, *i.e.* with acids or alkalis or by poisoning with metallic ions such as Ag^+ or Cu^{++} , their activity is destroyed equally both for bulk and surface reactions; on the other hand, when they are treated by boiling or the protein removed by digestion by another enzyme, only the bulk activity is impaired.

The interpretation advanced for these experiments revealing the peculiar relationship existing between the activity of what has been designated the "free enzyme" and proteins when presented to the "free enzyme" in the form of an orientated monolayer is based upon the hypothesis that the changes in phase boundary potential observed when a solution of an enzyme is injected under the surface of a buffer solution covered with a protein monolayer can be taken as a measure of enzymic activity, *i.e.* a digestion of protein by the enzyme. In

support of this hypothesis may be advanced the results of the investigations described in Part I where the process of gradual liquefaction of a protein film by an enzyme was described, the coincidence of the p_H optima for both bulk and surface reactions, the fractional changes effected by the proteinase and carboxypolypeptidase respectively, and lastly the various forms of what have been termed velocity curves obtained with different concentrations of enzyme, which curves are faithful replicas of the velocity curves obtaining for bulk solution in these systems.

It seemed desirable to show more directly that the disappearance of a protein film from the surface of a buffer solution after injection into that solution of a proteolytic enzyme was in reality a digestion of the protein.

A solution containing both egg-albumin and pancreatin at p_H 8 was allowed to undergo digestion. From time to time a few drops of the solution were placed by means of a dropping pipette on the surface of a buffer solution at p_H 8 and the change in phase boundary potential noted. The phase boundary potential in all cases attained the characteristic value obtained for the original protein with the formation of a solid film until complete digestion had taken place as determined by the absence of a precipitate with acetic acid, when the value characteristic of solutions of trypsin would appear and no solid film be formed. Evidently undigested protein in the drops spreads on the surface in preference to the trypsin or the products of hydrolysis until no protein is left in the solution. The identity of the phase boundary potential with that of the protein reveals no evidence of a protein-trypsin complex, although, as will be noted in the next series of experiments, evidence for the existence of a complex between some constituent in the pancreatin and a breakdown product of egg-albumin can be presented.

In the next series the trough was filled with buffer solution containing pancreatin and egg-albumin in various concentration ratios and the phase boundary potential determined from time to time as the process of bulk digestion proceeded.

It was found that the phase boundary potentials shown by these solutions were very sensitive to the ratio of the pancreatin to albumin. When the original solution contains a slight excess of pancreatin to protein the surface of the solution is found to acquire a solid film rapidly with all the characteristics of the protein, but this liquefies in the manner described in the investigations on monolayers, and within a few minutes the phase boundary potential acquires the characteristic value of a pancreatin solution (Fig. 11, graph *a*).

In experiments in which equal proportions of pancreatin and egg-albumin are present (0.1 % of each in the solution), the surface rapidly acquires a solid protein film with the characteristic phase boundary potential for egg-albumin (graph *b*); this falls slowly as the concentration of the protein diminishes until what must be regarded as a complex of trypsin and a product of hydrolysis of the protein commences to penetrate the surface layer, gradually increasing in quantity as the protein diminishes until ultimately the surface consists entirely of the trypsin complex possessing phase boundary potentials dependent upon slight variations in the original ratio of enzyme to protein, in general much higher (*e.g.* 178 mv.) than that of a trypsin solution (90 mv. at p_H 8). The surface is, however, perfectly fluid and no change takes place in the phase boundary potential on contracting the surface area by means of a slide. In the last case where the solution contains an excess of protein to pancreatin the surface reaction evidently becomes more complicated (graph *c*). It is observed that the protein film of phase boundary potential 250 mv. forms immediately as in the other cases. The phase boundary potential falls in twenty minutes to 193 mv. and then com-

mences to rise again. The originally rigid gel-like film decreases in rigidity, becomes more elastic and then eventually becomes rigid again. At the end of 220 minutes the original protein-like characteristics of the surface film have all returned.

These films are, however, ultimately digested away after prolonged periods, *ca.* 10–15 hours.

Fig. 11. Surface potential of bulk phosphate at p_H 7.7. *a.* 0.3 g. trypsin + 0.2 g. egg-albumin in 300 cc. at 25° . *b.* 0.3 g. trypsin + 0.3 g. egg-albumin in 300 cc. at 25° . *c.* 0.1 g. trypsin + 0.2 g. egg-albumin in 300 cc. at 25° .

The simplest interpretation to be placed on these observations is that after a preliminary partial digestion of the surface film has taken place the products and the enzyme present near the surface are displaced by the large quantities of undigested protein in the bulk phase and the regenerated protein film then persists until the concentration of the protein in the bulk phase has been greatly reduced.

Further experiments were carried out to test the effects of known by-products in the pancreatin (lipase and amylase) on a protein monolayer.

A pancreatin solution was filtered at p_H 4.6 and the filtrate treated with alumina B. The elution from the alumina containing the lipase and amylase did not influence the surface potential of a caseinogen film, whereas the residue containing the tryptic ferments displaced the caseinogen film in the usual manner.

It may be concluded from these experiments that the change in phase boundary potential and disappearance of a protein film may be taken as an indication that proteolytic hydrolysis or digestion is in reality proceeding.

SUMMARY.

It is shown that when solutions of pancreatin are injected under monolayers of proteins such as egg-albumin or caseinogen proteolytic hydrolysis of the latter proceeds. The reaction can be followed qualitatively visually and quantitatively by observing the concomitant changes in phase boundary potentials.

The reaction kinetics of the proteolytic digestion of protein monolayers follow closely those previously observed for the bulk phase reaction both in respect to dependence on the p_H of the solution and the order of the reaction. It is shown that the proteinase fraction of pancreatin is responsible for the removal of some 10 % of the protein in the case of egg-albumin and of 15 % in the case of caseinogen, whilst the carboxypolypeptidase fraction removes a further 25 and 16 % respectively.

The proteinase and carboxypolypeptidase fractions of pancreatin when purified by the precipitation or by the adsorption process form films which are protein-like in character. These are themselves capable of being hydrolysed by pancreatin without loss of surface activity.

The characteristics of films of trypsin are found to be indistinguishable from those of albumin over the region of p_H in which they exhibit no tryptic activity, whilst over the active region they form solutions of low surface phase boundary potentials.

It is shown that when pancreatin solutions are digested by pepsin at p_H 2 and the solution restored to p_H 8, whilst no tryptic activity is observed for bulk phase reaction, monolayers of egg-albumin and caseinogen are readily digested. Solutions of trypsin when boiled at p_H 8 likewise exhibit a marked surface proteolytic activity.

The properties of the surfaces of solutions containing both enzyme and protein are examined during the course of the reaction. It is concluded that in the tryptic enzymes the "free enzymes" can be separated from their protein-like carriers but when free can only react with proteins when presented to them in a suitable form such as a monolayer at an air-water interface. To render the "free enzyme" active for protein solutions it must be adsorbed on a protein in a particular state or configuration. Experimental evidence is given that the disappearance of a protein film on an enzyme containing substrate is due to digestion only and is not due to the adsorption of some constituent of the enzyme of lower surface tension.

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