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Proteins and Enzymes

By

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III. THE INITIAL STAGES IN THE BREAKDOWN OF PROTEINS BY ENZYMES

My lecture today will be on a subject which is rather far removed from those discussed in my first two lectures. When one is interested, however, in what happens in biological systems, it is extremely valuable to know something about the structure of their proteins. These substances are the most important ones in the cell, since they form the structural elements, with the enzymes catalyzing chemical reactions. Among the processes of enzymatic character that I have been most interested in, in the past years, is the breakdown of proteins by proteolytic enzymes. It is naturally an extremely complicated reaction. Considering how little we know about the mechanism of the enzymatic breakdown, even of much simpler substances, we must be very cautious in the interpretation of the results on the breakdown of proteins. It is therefore natural that during the past years many workers have tried to find simple substances which are cleaved by the same proteolytic enzymes that attack proteins. A great deal of very important investigations have been carried out by numerous authors, starting with the early work of Emil Fischer. I may mention work by E. Abderhalden, by the Willstätter school, and especially by Max Bergmann and his co-workers, who have contributed much to the solution of the question of what determines the specificity of proteolytic enzymes.

As matters stand at the moment, we must assume that in proteins as well as in their breakdown products, peptides, etc., the central point of attack for proteolytic enzymes is the peptide bond, which is split as follows in aqueous solution:

$$-\mathrm{CO}-\mathrm{NH}-\xrightarrow[\nearrow]{\text{Enz, } \mathrm{H_2O}}-\mathrm{COO^-}+\mathrm{NH_3^+}-$$

Although important work by Neurath and his school (1) has widened the range of specificity of proteolytic enzymes to include the amino acid ester bond, we can safely assert that the peptide bond is the principal "substrate" for these enzymes.

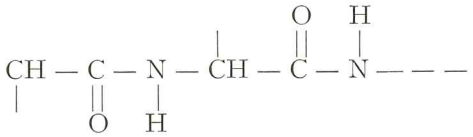
I shall not have time to go into other specificity-regulating factors in peptides, such as the influence of electric charges (COO^- or NH_3^+ groups) proximal to the bond split, or the influence of the amino acid side chains, both factors that determine which bonds can ultimately be split in a peptide by a given proteolytic enzyme.

A more directly relevant question is the following: Whereas the enzymatic opening of peptide bonds in simple peptides is a phenomenon which may be adequately described (if not explained) on the basis of our knowledge of the physicochemical properties of both substrates and reaction products,

numerous observations have shown that the chemical reaction by which the peptide bond is opened in proteins is accompanied by more or less obscure physical (or chemical) processes involved in the dramatic breakdown of the giant molecules. It is my purpose today to discuss certain aspects of this problem.

You know that Fischer's original idea was that proteins consist of peptide chains built up by amino acids. Ever since the first organic chemical study of proteins, this hypothesis has been the simplest one. The difficulty was only that it was hard to prove with the methods available, and that, therefore, several other hypotheses for the structure of proteins were put forward, e.g., Abderhalden's diketopiperazine ring hypothesis, D. M. Wrinch's cyclol theory, and other very stimulating but transient theories. We have now returned, I believe, to Fischer's theory and consider the proteins as being built up by peptide chains. At any rate, that is what I am going to assume today.

However, proteins do not behave, in aqueous solution, as simple peptide chains. Even in the more simple proteins—the fibrous proteins, for instance —there is a superstructure involved, by which I mean that the peptide chains are not free to move in all directions but are bound either internally or to other peptide chains to form structures of greater stability and less flexibility than the free peptide chain. Hence, the first question is: What kind of forces interlink these peptide chains? It is self-evident that we must look for an answer to this question in the properties of the chains, both the properties of the "backbone":



and those of the characteristic side chains of the amino acids, some with ionizable groups, some with hydrophobic groups, and some with hydrophilic but not ionizable groups (hydroxyl groups in serine). The second question is: What does the detailed superstructure of the giant molecule look like, in which these forces have combined peptide chains with a certain pattern of amino acids? These two questions are, of course, ultimately connected since the forces must depend upon the structure.

In recent years, the study of the X-ray diffraction pattern of protein crystals has given very important information about the arrangement of the peptide chains in proteins. I do not consider it my job here to try to give a survey of these investigations, but I should like to point out that through the work of Meyer and Mark, Astbury, Huggins (2), the Bragg school, Bernal and Perutz for instance (3), Pauling and Corey over here (4, 5), we have at the moment got an indication or perhaps more than an indication of how these peptide chains are arranged in certain protein molecules. This is particularly true for fibrous proteins—α-keratin and other proteins of this type—the structure of which has been satisfactorily explained by the Pauling-Huggins-Bragg theory, while in the case of globular proteins—hemoglobin, albumins, and globulins, for example—our knowledge is considerably more uncertain. For my primitive and highly qualitative considerations, however, it is of relatively small importance whether or not there is a complete fit between deduced structure and experimental evidence if only the general picture is true. Assuming this, I shall therefore make a sketchy drawing of the structure of a globular protein molecule, based essentially upon the recent theory of Pauling and Corey. I hope by means of this model to bring out characteristic features of the enzymatic attack upon the molecule.

According to Pauling and Corey's theory, the peptide chains form spirals or helixes within the protein molecule. These helixes are stabilized by internal hydrogen bonds between CO and NH groups along the backbone so that all the CO and NH groups are involved, except the first three NH groups and the last three CO groups, in a finite, open peptide chain. The number of amino acid molecules per turn of the helix is 3.7. In this structure the side chains of the amino acids are all extrovert and, because of the fact that there is not a whole number of side chains per turn, they are seen to be arranged in steep spirals around the core of the backbone helix. Another structure is also proposed by Pauling, in which the helix has 5.1 amino acid molecules per turn, a considerably looser one in which the numbers of unbound NH and CO at the ends are 5. At the moment I do not think that we are able to decide between these two possibilities.

In the globular protein molecule we must now assume that these helixes are bound together to form nonlinear structures either by end-to-end or, especially, by side-by-side association. The end-to-end association will be in part electrostatic, that is, initiated by the attraction between the terminal NH_3^+ group in one chain and the terminal COO^- group in another. Furthermore, there is the possibility of a stabilization by hydrogen bonding between the terminal free NH and CO groups.

The side-by-side association must be determined almost exclusively by the side chains of the amino acids, since there is little possibility for cross-linkage by hydrogen bonds between the backbone atoms. In many globular proteins the number of terminal amino and carboxyl groups is very small so that, if the helixes were unbent, the shape of the molecule would be much more rodlike than found by physical measurements. We have to assume, therefore, that there are cross-connections between the ends of the helixes where the backbone peptide bonds are exposed and not involved in hydrogen bonding within the helix. Bonds of this type are shown in Figure 38 at *X* and *Y*. The existence of such straight pieces of peptide chain will increase the possibilities of structural variation from protein to protein. However, the

frequency of cross peptide linkages and the average lengths of the helixes in proteins are not known; nor can we evaluate Pauling's suggestion that the amino acid proline, whose nitrogen group, when peptide-bound, lacks the hydrogen required for hydrogen bonding, is always involved in such cross-linkages.

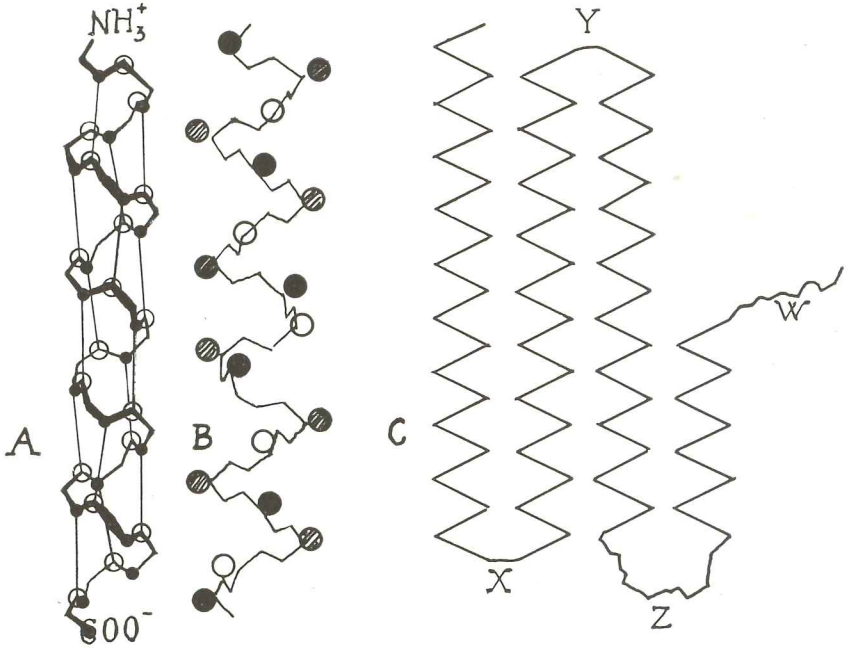


FIG. 38.—*A*, helix, backbone with hydrogen bonds. ○ NH groups; • CO groups. *B*, same helix, showing position of side chains. ● in front; ○ behind. *C*, folded peptide chain with helixes, cross peptide bonds, a loop, and a tail.

In order to complete the picture, I shall discuss the side-by-side association of helixes. There are here a number of different kinds of bonds that must be taken into account.

First of all, there is the chemical bond, for instance, the SS bond in cystinyl residues, so important for the interlinkage of peptide chains in insulin and keratin, or further, the peptide bond between carboxyl groups and amino groups of the side chains. The γ-carboxyl group of glutamic acid and the ε-amino group of lysine may form such a bond. This possibility is, so far as we know, realized in very few proteins.

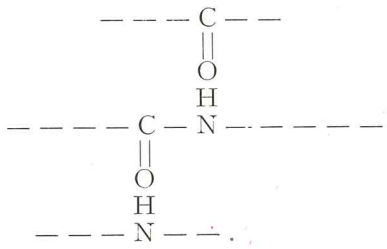
Then we have the so-called salt linkages, which, if existing exposed to water, must simply be regarded as electrostatic attractions between the negative and positive charges of the hydrated ionized groups of the side chains.

Every time a COO^- group from glutamic or aspartic acid in one helix approaches a NH_3^+ group from lysine or arginine in another we may say that a salt linkage is being formed. If the pattern of positively and negatively charged ions is such that many ion pairs of this sort can exist simultaneously, rather considerable forces may arise and may serve to keep the molecule together.

In some cases we may imagine that the ionizable groups are concealed in the interior of the molecules and are pairwise matched COO^- to NH_3^+ to form hydrogen-bonded structures (6, 7, 8) such as are found in amino acid crystals (5, 9). The necessary condition for the establishment of these very strong bonds is protection against water, and there is some evidence that such bonds are rare in globular proteins (10). I shall return to this question in a moment.

Finally we have van der Waals' forces, especially between the hydrophobic side chains of the amino acids alanine, valine, leucine, or isoleucine. These may unite just as the carbon chains in soap micellae unite to form stable structures.

Hence, a number of different forces are involved in the building up of a protein molecule. One may consider each molecule as a small crystal (11) because it is actually bound together by exactly the same forces as you find in crystals of amino acids and peptides, one factor being accentuated because of the difference in size—the ionizable groups like COO^- or NH_3^+ seem to be free in most proteins to react with hydrogen ions. There are, however, exceptions (Steinhardt, 12); and as regards reactions with chemical reagents having larger molecules than the hydrated hydrogen ion, many examples are known of partial or complete concealment, masking, of otherwise reactive groups in the side chains—SH, tyrosine, lysine groups (15, 14). In most cases this masking is due to steric hindrance, but chemical bonding may be involved, too. The relative stability of many native proteins toward proteolytic enzymes, a stability that they lose upon denaturation, may be explained as being due to the masking of peptide bonds in the helix



Besides this characteristic general structure, the protein molecules may show some individual features that cannot be classified under the previous headings. Longer "tails" at amino ends or carboxyl ends of the peptide chains

or longer intermediary pieces (loops) may not be involved in intrahelix bonding (see Fig. 38). I shall return to this possibility in my next lecture.

In the beginning I used the word superstructure to name the structural features other than those characterizing the unfolded peptide chain—the so-called β-chain (Astbury). The presence of intrahelix as well as interhelix bonds may justify a classification into secondary (intrahelix) and tertiary (interhelix) structures, as distinct from the primary structure of the simple β-chain. I shall use these names in the following.

Before going into the enzymatic breakdown of the protein molecule I shall briefly discuss one typical and often reversible process of nonenzymatic character that many proteins undergo in aqueous solution, namely, denaturation.

What happens to the protein molecule when it is denatured? We know that the proteins, when uncharged (at the isoelectric point), will lose their solubility in water or salt solutions. We know that certain groups of the side chains such as SH, tyrosine, and lysine groups become unmasked, and that the shape of the molecules changes to a more fibrous type. At the same time the volume of the molecule decreases (11) and there is a strong increase in optical rotation (15, 16, 17), showing that groups adjacent to the asymmetric carbon atoms in the backbone are involved. On the basis of our model it is reasonable to assume that both the secondary and tertiary structures are involved in the process of denaturation, that the helixes unwind wholly or partially, and that the weaker interhelix bonds are broken without change in the primary structure. In certain cases where the unfolding is not too extensive, the different parts of the molecule may find their way back to their original positions so that the denaturation is reversible (Anson and Mirsky). In other cases this may not be possible because recombination in other and more irregular patterns has occurred. Precipitated, denatured proteins are typical in this respect, probably because of interlinkage between different peptide chains by means of hydrogen bonds (the "pleated sheet" formation according to Pauling, 5).

Now after this rather long introduction serving to give a picture of the protein molecule, I shall discuss the main issue, namely, the breakdown of this molecule by enzymes. What can we imagine will happen when the molecule is attacked by an enzyme?

I said in the introduction that the chemical reaction by which the peptide bond is opened in proteins is accompanied by other physical and chemical processes involved in the disintegration of the giant molecule. It is quite clear from our model that in the enzymatic process, on the way down to lower peptides, the structures that we have termed secondary and tertiary must become abolished. Hence, there must be an element of denaturation in every exhaustive enzymatic cleavage of proteins. The question that has interested me particularly is at what stage the denaturation occurs and what its

rate is, compared with that of the splitting of peptide bonds in the primary structure. Furthermore, there is the question whether the proteolytic enzymes attacking proteins, the proteinases—besides displaying their action as endopeptidases (Bergmann) capable of splitting peptide bonds in the backbone of the protein chains—are also capable of directly denaturing the proteins they attack, either completely or locally around the bonds they subsequently cleave as endopeptidases.

Unfortunately no general answer to these questions is possible, because different proteins and enzymes behave differently and even the same protein-enzyme system may be changed by varying the external conditions. I shall therefore limit myself to outlining a few typical cases.

I shall first consider the possibility that the enzyme does not denature the substrate protein directly.

The simplest but most unlikely assumption here is that all the specific bonds that can be cleaved by the enzyme as an endopeptidase are accessible to it in the native molecule and that this falls apart gradually as the bonds are opened. In this case denaturation occurs in small steps during the chemical reaction and is difficult to characterize as such.

A more likely reaction scheme in which spontaneous denaturation may be determining for the initial step in the cleavage of proteins is the following (18): If at the conditions of the enzymatic experiment the substrate protein is capable of denaturing spontaneously and reversibly, we must have an equilibrium between native and denatured molecules in the solution

$$N \rightleftarrows D.$$

If, therefore, the denatured form, D, with its exposed peptide bonds, is split by the enzyme and removed from the equilibrium mixture, the process $N \rightarrow D$ will be pushed from left to right and the native form N will gradually disappear.

A further possibility is that the enzyme attacks the substrate molecule at certain spots, which are more or less open toward the outer world. It is reasonable to assume that, although the majority of peptide bonds in our model would be hard for the enzyme to attack because of the stability caused by hydrogen bonding, the exposed cross peptide linkages X, Y, or Z may be easier both to approach and to split. If such bonds are broken, the reaction would possibly make the molecule unstable, so that it would denature spontaneously whereby new peptide bonds might be exposed to the endopeptidase action. At the present moment this possibility cannot be excluded. I may also mention that the splitting of bonds like X, Y, Z, or W without further extensive degradation may be involved in such well-known processes as the activation of zymogens, where the protein molecule apparently is very little changed chemically but where, anyhow, peculiar new properties are created. I shall return to this question in a moment.

The possibility that the enzyme denatures the substrate protein directly must be considered next.

In this case, as in the previous one, the initial step may be a cleavage of a few exposed peptide bonds of the type X, Y, or Z. But it is assumed that the substrate and enzyme proteins come into so intimate a contact that the secondary and tertiary structures of the substrate are destroyed, at least temporarily, so that endopeptidase activity can set in. Perhaps a denaturation occurs in both the enzyme and the substrate molecule, that of the enzyme being a reversible one. We may use the picture of two boxers in the ring who go into a near-fight: one of them breaks loose from the clinch intact and ready for a renewed attack, but the other is badly shaken and at the mercy of the first for the rest of the fight. The picture is naturally very rough, but in the absence of more definite ideas about the mechanism of this protein-protein interaction it may be excused. All we can say is that, during the contact, intermolecular bonds of the same type as the intramolecular bonds in the separate proteins may be formed temporarily, causing instability in the substrate molecule.

In the following paragraphs I shall give two typical examples that may serve to illustrate experimentally the conjectural considerations of the first part of my lecture.

Figure 39 shows two curves representing the activation of chymotrypsinogen by trypsin. The experiments were carried out by Jacobsen at the Carlsberg Laboratory (19). The two curves correspond to two different enzyme concentrations, their ratio being one to thirty-six. The ordinates are the activity as percent of the "potential activity," which is the highest activity obtained with the concentration of chymotrypsinogen used. The abscissae are the times. The activity was measured by Kunitz' method.

Figure 40 shows some theoretical curves which have been calculated by Jacobsen on the basis of the assumption that a series of consecutive reactions occurs in the activation process. The first of these reactions is catalyzed by trypsin and transforms chymotrypsinogen into π-chymotrypsin, which is 2.25

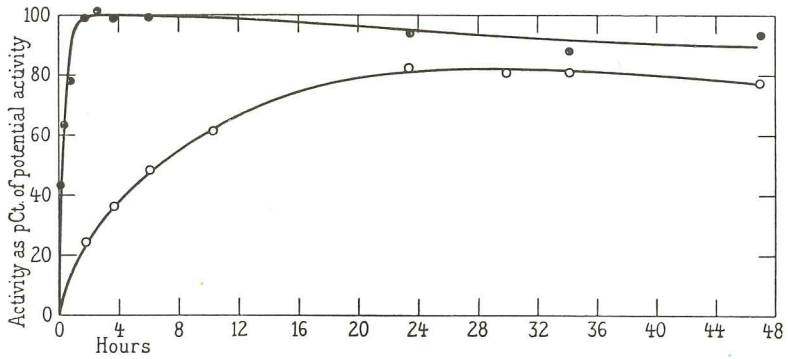


FIGURE 39

times as active as Kunitz' well-known enzyme α-chymotrypsin. π-Chymotrypsin is then again by trypsin (or possibly by itself) transformed into δ-chymotrypsin, which in turn by autocatalysis is broken down to α-chymotrypsin. δ-Chymotrypsin is 1.5 times as active as α-chymotrypsin. As in Figure 39, the different curves in Figure 40 correspond to different trypsin concentrations. A comparison of Figure 40 with Figure 39 reveals the similarity between experimental and theoretical results.

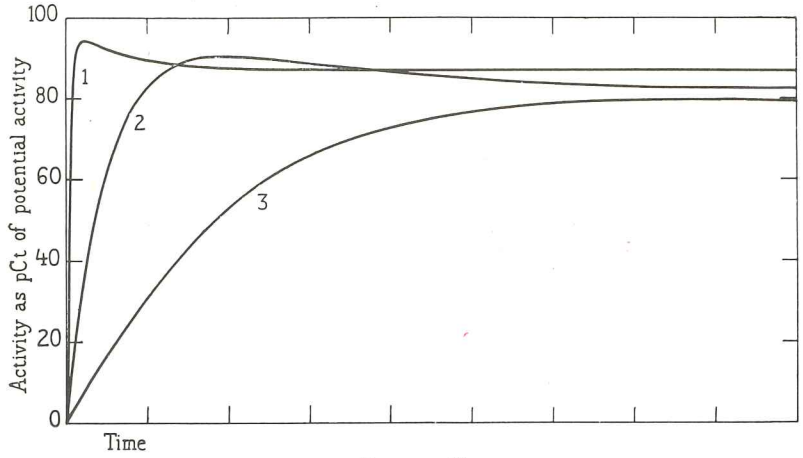


FIGURE 40

In the formation of π-chymotrypsin, apparently only one peptide bond in the chymotrypsinogen molecule is split by trypsin. As appears from Figure 41, there is an excellent agreement between three experiments showing the relation between activity, as percent of the potential activity, and the number of peptide bonds split per molecule as determined by titrations in acetone (and alcohol). You will observe that the initial slope is such that the tangent at the zero point will cut the axis of abscissae corresponding to 100 percent activity a little below one peptide bond. However, the potential activity is lower than the specific activity of π-chymotrypsin because this enzyme has a short lifetime and is almost completely transformed into δ-chymotrypsin when the potential activity is reached. If, therefore, we move the axis of abscissae upward above 100 percent activity, say to the ordinate

$$\frac{\text{Spec. activity } \pi\text{-chymotr.}}{\text{Spec. activity } \delta\text{-chymotr.}} \times 100 = \frac{2.25}{1.5} \times 100 = 150 \text{ percent},$$

we get intersection at one peptide bond. Hence, we have here a very simple process where apparently only one peptide bond is split in a protein molecule and yet this molecule acquires a catalytic activity, becoming a powerful proteolytic enzyme, π-chymotrypsin.

Naturally it cannot be decided whether the chemical change observed is the only one that occurs, and so we cannot with certainty classify the reaction as being of the type in which only bonds like X, Y, Z, or W (Fig. 38) are involved; nor can we explain why the splitting of such bonds makes the molecule enzymatically active. It is quite conceivable that the cleavage of one peptide bond will make the chymotrypsinogen molecule unstable, but that via a partly denatured state, it may rearrange to a more stable "native" configuration characteristic of π-chymotrypsin.

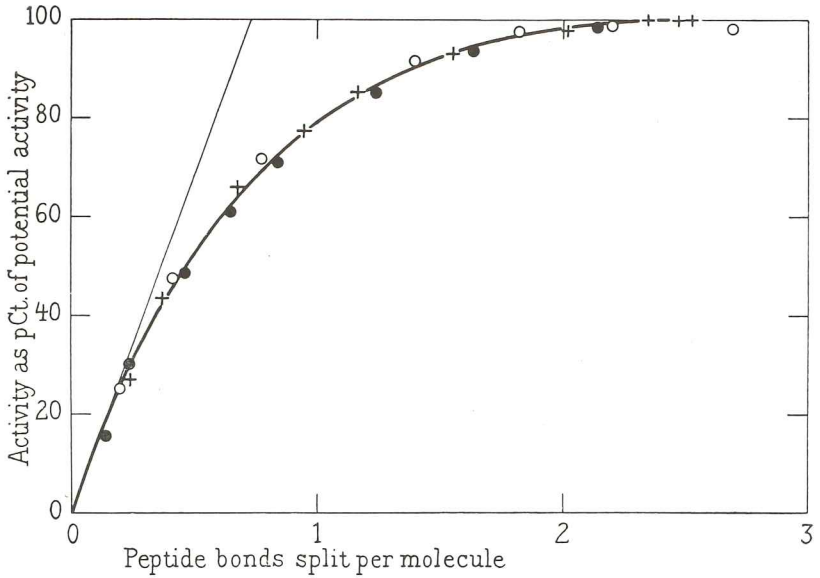


FIGURE 41

In order to throw some light upon this question, Jacobsen has measured the volume change accompanying the splitting of the first peptide bonds in the transformation of chymotrypsinogen into π-chymotrypsin and further into δ-chymotrypsin (Fig. 42). This volume change, which is measured by a simple dilatometric method, is about 30 ml. per mole peptide bond split and therefore considerably greater than would be expected if nothing else happened than the opening of the bond. In a moment I shall talk a little about what that means.

When finally I turn to the question of the rate of denaturation in the deeper enzymatic breakdown of proteins, I shall use as an example the tryptic degradation of β-lactoglobulin and report some investigations carried out in the Carlsberg Laboratory by Jacobsen, Johansen, Korsgaard Christensen, and me (11). Other systems of this type will be discussed only when

necessary for understanding the problem in hand. In our theoretical considerations we are indebted to the Williams school (20), especially Lundgren (21), and to W. Kauzmann.

In order to follow the denaturation simultaneously with the cleavage of peptide bonds in the breakdown of proteins, some property which is inherent in the protein molecule and is changed by denaturation must be measured along with the liberation of carboxyl or amino groups (followed by suitable titrations). We have selected two properties, the apparent volume and the

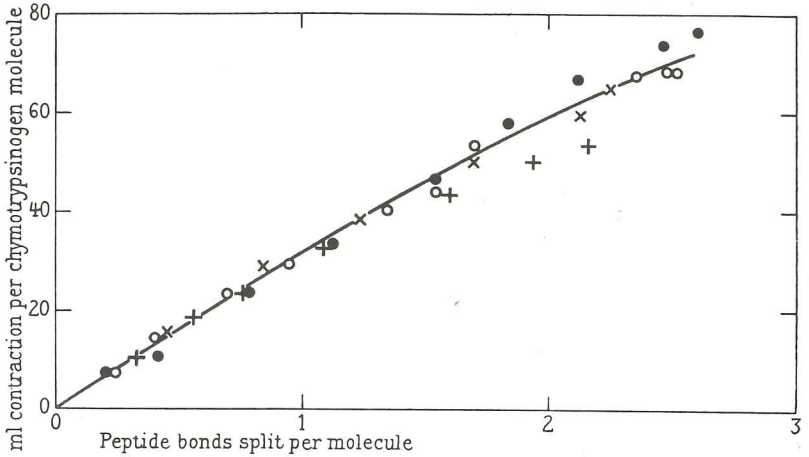


FIGURE 42

optical rotation. Although both properties will change owing to the cleavage of peptide bonds alone, the changes involved here may be distinguished from those due to denaturation on the basis of differences in magnitude or sign.

When a peptide bond is split in the middle of a long, straight peptide chain, experiments by Weber and Nachmanson and Cohn and co-workers have shown that the cleavage will be accompanied by a volume decrease, a contraction, of about 20 ml. per mole, which is essentially due to the electrostriction around the carboxylate ion and the ammonium ion formed. The water molecules are attracted by these ions, and the water is therefore in a compressed state around them. A detailed investigation (22) of this question has led to the conclusion that in the *p*H range 6–10, the value 20 ml. is a maximum one for simple polypeptides. However, in the case of proteins of the globular type much higher volume changes are actually found, and we ascribe this to the collapse of the secondary and tertiary structure of the protein molecule. There is, in fact, a contraction in denaturation which is of a sufficient order of magnitude to explain the phenomenon, and in the case of already denatured globular proteins the volume decrease in proteolysis ap-

proaches the theoretical value of 20 ml. per mole peptide bond split. Hence, in the excess volume change in proteolysis we have a possible measure of the denaturation which precedes, accompanies, or succeeds the cleavage of peptide bonds. At the present moment we do not know enough about the physical properties of the helixes and their interlinkages to determine with certainty the origin of this extra volume decrease. The unfolding of a 5.1 helix may give a contraction, but disruption of salt linkage or separation of associated hydrophobic groups may also be involved.

In the case of optical rotation, denaturation gives a marked rise in the levorotation of proteins (15, 16, 17, 23). The effect is dependent upon *p*H. Proteolytic breakdown of a fully denatured protein results in a decrease in levorotation, while the splitting of native protein is accompanied by a rise in levorotation. Again this physical property may be used as a measure of denaturation in proteolysis. As pointed out by Kauzmann, the marked change in rotation upon denaturation indicates that the helix structure in which the backbone of the peptide chains is involved, and which contains the asymmetric carbon atoms, must be changed in this process.

The picture of the tryptic breakdown of β-lactoglobulin that I shall give next will be based on the following assumptions, which are admittedly too simple but which are to some extent borne out by the experimental data.

The initial process is either a denaturation without preceding cleavage of peptide bonds (but possibly with a simultaneous cleavage of a few bonds), or a denaturation after cleavage of very few peptide bonds per molecule. In both cases the denaturation must be essentially irreversible in the sense that if the trypsin molecule dissociates from the substrate molecule after the initial attack, the latter is unable to return to the native state. The further deep-going breakdown of the denatured substrate is comparable to that of a fully extended peptide chain, its ultimate limit being determined by the specificity of the enzyme as an endopeptidase. I shall term these two processes the initial and the endopeptidase reactions.

Now, as pointed out by Tiselius (24), there are two possible extremes in the way in which a proteinase can attack a protein. It may split all the possible peptide bonds in each substrate molecule before it goes to the next. This reaction type I shall call the "one-by-one" type. Or the enzyme may act in such a way that it runs from molecule to molecule and breaks, for instance, one peptide bond in each before it starts to cleave another bond in each, etc. This reaction type may be called the "zipper" type. All naturally occurring proteolytic reactions may be considered intermediates between these two extremes.

It is easy to see that in our particular problem the relative rates of the initial and the endopeptidase reactions determine the type of the composite reaction (11). If the initial reaction is infinitely slow relative to the endopeptidase reaction, each substrate molecule will be extensively degraded

before the enzyme attacks the next and the reaction will be of the one-by-one type. The reaction mixture will contain only unchanged substrate molecules and final reaction products. If, therefore, the volume change or the optical rotation is plotted against the number of peptide bonds split, a straight line will be obtained, represented, for example, by the equation

$$\Delta V = (V_i + n\,V_E)\,X = \left(\frac{V_i}{n} + V_E\right)\Delta P$$

where ΔV is the observed volume change, V_i and V_E the volume changes in the initial and the endopeptidase reactions respectively, n the number of peptide bonds that can be cleaved in a substrate molecule, X the number of molecules degraded, and ΔP the observed number of peptide bonds split ($= n \times X$). (See Fig. 43.)

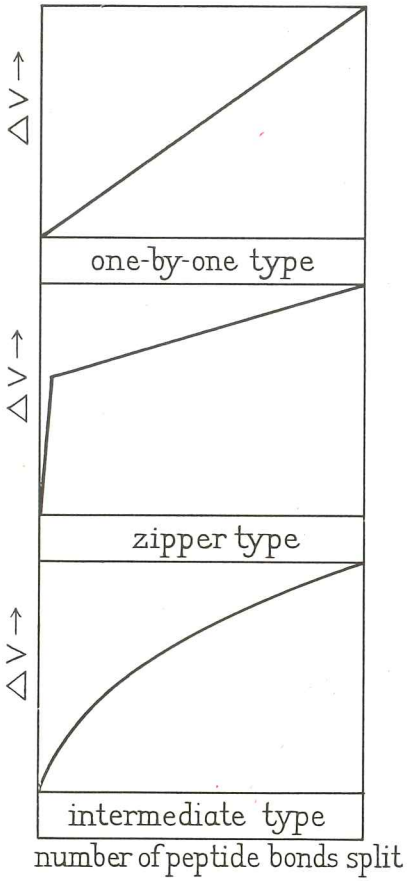


FIGURE 43

If the initial reaction is infinitely rapid in comparison to the endopeptidase reaction, all the substrate molecules will initially be denatured by a process of the zipper type and with a small cleavage, or no cleavage, of peptide bonds. Afterward the exposed peptide bonds will be split slowly and at random in the unfolded chains, so that a great number of intermediary peptides of different length will be formed during the degradation. The initial volume change per peptide bond split will be very great or infinite while in the second stage $\Delta V/\Delta P = V_E$ will be around 20 ml.

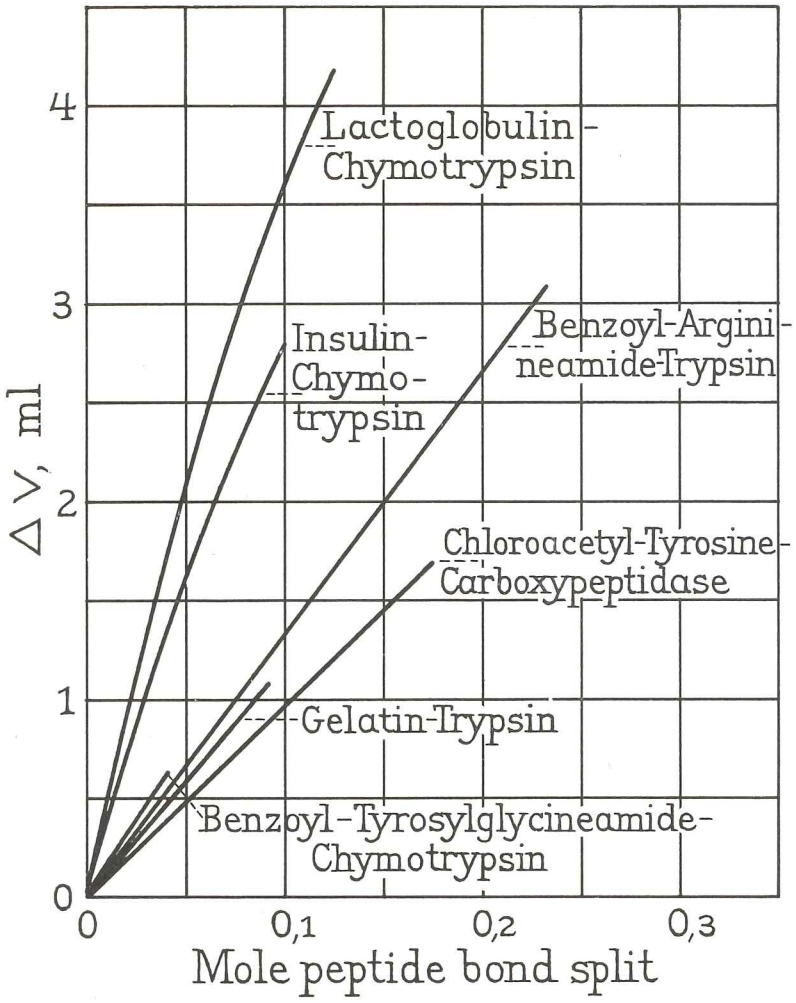


FIGURE 44

In intermediary cases $\Delta V/\Delta P$ will decrease gradually with time and smooth curves will be obtained. Intermediary peptides will occur.

Figure 44 shows some curves for the volume change in the splitting of different proteins with different enzymes. You may observe that the curves for globular proteins represent high volume changes and are more curved than those for simple peptides or peptide-like substances which are straight lines and correspond to smaller volume changes.

Figure 45 shows a series of curves for the splitting of β-lactoglobulin

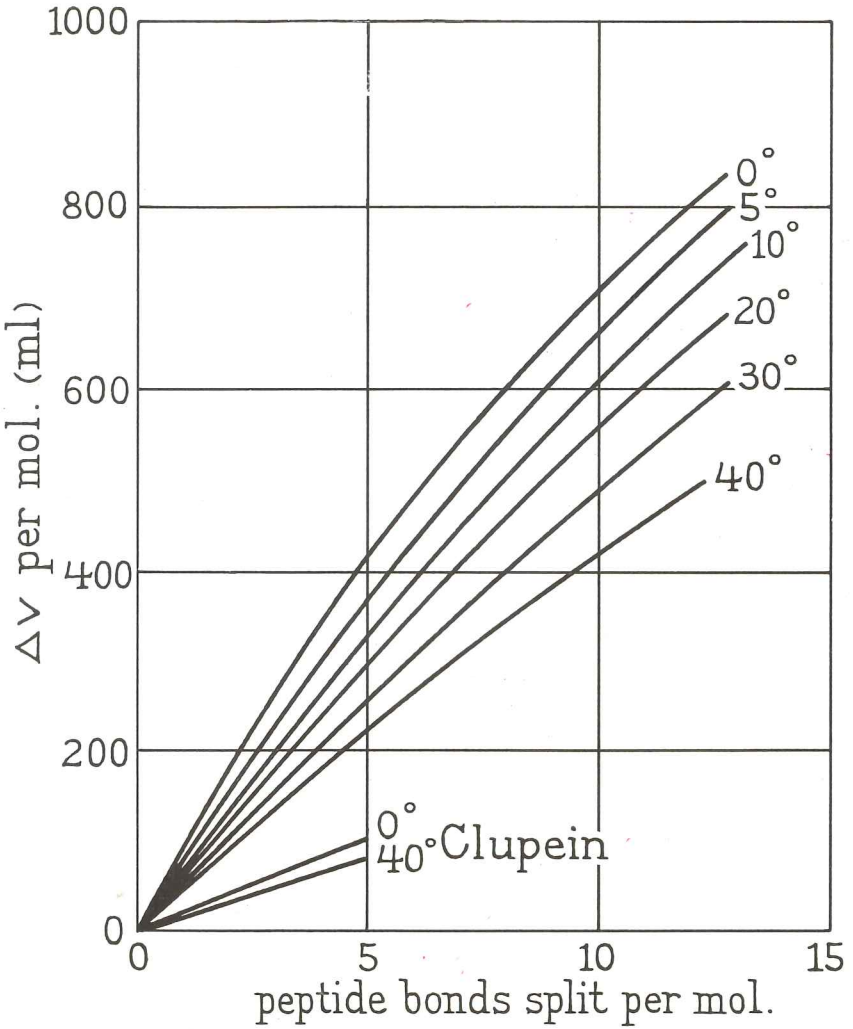


FIGURE 45

by trypsin at different temperatures (11). It demonstrates the peculiar fact that there are great differences in both the shape of the curves and the magnitude of the volume change at different temperatures. In terms of my previous considerations these experiments indicate that, with increasing temperature, the system approaches the one-by-one type, $\Delta V/\Delta P$, being practically constant at 40° C. At 0° the process is more of the zipper type; the initial $\Delta V/\Delta P$ is very large (100 ml/mole peptide split), and intermediary products may be expected to appear. We may therefore conclude that the initial process necessary for the opening of the β-lactoglobulin molecule for the attack of the endopeptidase is relatively more rapid at 0° than at 40°. Now what is it that makes the relative rate of denaturation greater at low temperature than at high temperature?

In order to see that, we must look at some figures showing the denaturation of β-lactoglobulin by urea. As is well known, urea is a substance which acts strongly denaturing upon many proteins, and in the case of β-lactoglobulin, as shown by Jacobsen and Korsgaard Christensen (25), the denaturation has a very high negative temperature coefficient (Fig. 46).

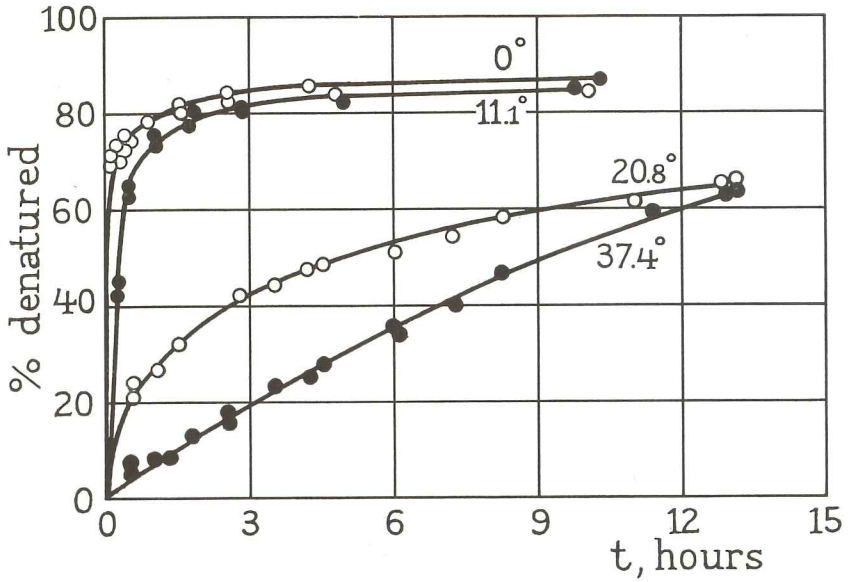


FIGURE 46

At 0° the rate of denaturation in 38 percent urea is extremely high, but with increasing temperature it decreases rapidly. Denaturation was followed here by determining the percentage of the protein which becomes insoluble at the isoelectric point.

Figure 47 shows the reversibility of the denaturation, measured in the same way (25). The concentration of urea is 38 percent and the temperature is varied from 0° to 37.4° C. The dotted curves represent experiments at 0° and 37.4°. If the solution at 37.4° is cooled to 0° right at the start, we get a high degree of denaturation. If then the solution is heated, it returns very rapidly to the conditions represented by the 37.4° curve, and if it is cooled and heated again, a similar rise and fall is observed.

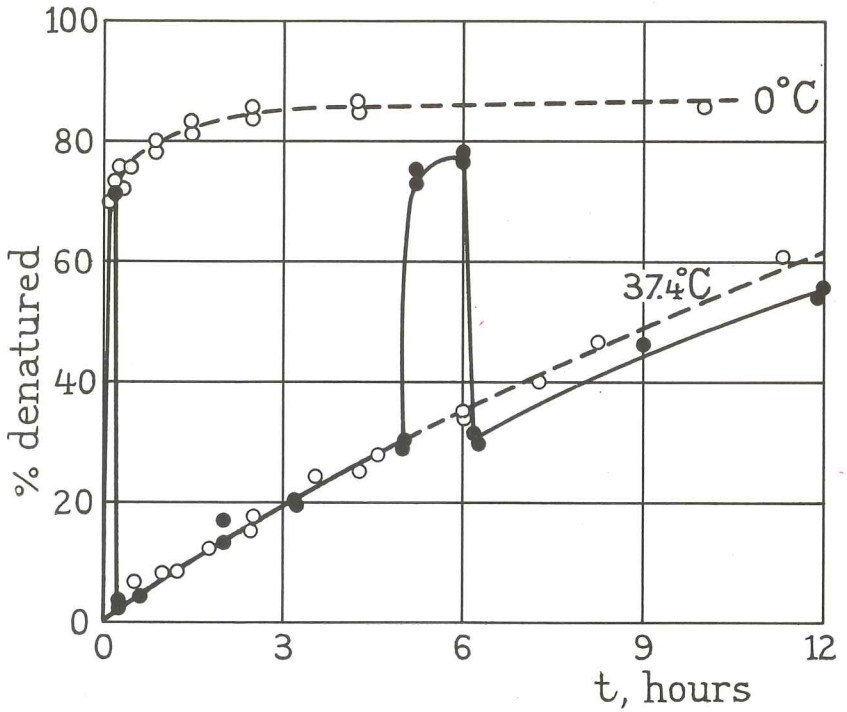


FIGURE 47

Figure 48 shows the same phenomenon as Figure 47, only here the denaturation is measured by the change in optical rotation (23).

It is evident, however, from Figures 47 and 48 that the reaction is complex, and a closer investigation has shown that one may distinguish between a reversible denaturation with a negative and an irreversible denaturation with a positive temperature coefficient. The latter is due in part to oxidation of SH groups liberated or unmasked in the reversible denaturation and is the faster reaction at 37.4° (Korsgaard Christensen, 23). The quantity of reversibly denatured β-lactoglobulin is therefore small here because it is continuously transformed into the irreversibly denatured protein.

We explain the negative temperature coefficient of the reversible denaturation by assuming that β-lactoglobulin exists in two forms L_s and L_i which are in mutual equilibrium. L_s is relatively stable and predominates at higher temperatures whereas L_i denatures easily and reversibly and predominates at lower temperatures. On this basis it is possible to draw theoretical curves that are very much like the ones in Figure 46.

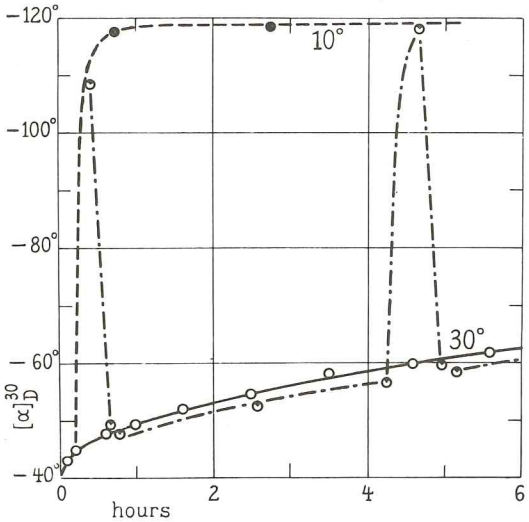


FIGURE 48

If we return again to the tryptic breakdown of β-lactoglobulin, it becomes clear that the assumption just mentioned is well able to explain the relatively high rate of the initial reaction at 0° where L_i predominates and where, therefore, the denaturation by trypsin is accelerated. That this enzyme is indeed capable of denaturing β-lactoglobulin either with or without preceding, simultaneous, or subsequent cleavage of few peptide bonds is shown in Figure 49, where are reported the results of a tryptic digestion of this protein in 19 percent urea at *p*H 8, 30° (Korsgaard Christensen, 26). The lower curve shows the rise in levorotation upon denaturation with urea alone. The three other curves show the striking effect of the addition of trypsin. Denaturation is accelerated by the enzyme and only at a higher concentration does another effect set in, namely, a decrease in levorotation which in parallel experiments with initially denatured β-lactoglobulin was shown to be the result of enzymatic breakdown, endopeptidase activity.

Very similar results were found by Korsgaard Christensen (23) in investigations of the denaturation of β-lactoglobulin in aqueous solutions at *p*H 9.3 without urea. Here, too, there was a marked accelerating effect by

trypsin (but not by heat-denatured enzyme). Korsgaard Christensen (23) has further been able to isolate considerable amounts of denatured protein—insoluble at the isoelectric point—from tryptic digests of β-lactoglobulin without urea, especially during the first part of the enzymatic reaction. It had practically the same molecular weight as β-lactoglobulin. So far it has not been possible to show that no peptide bonds were cleaved in this material.

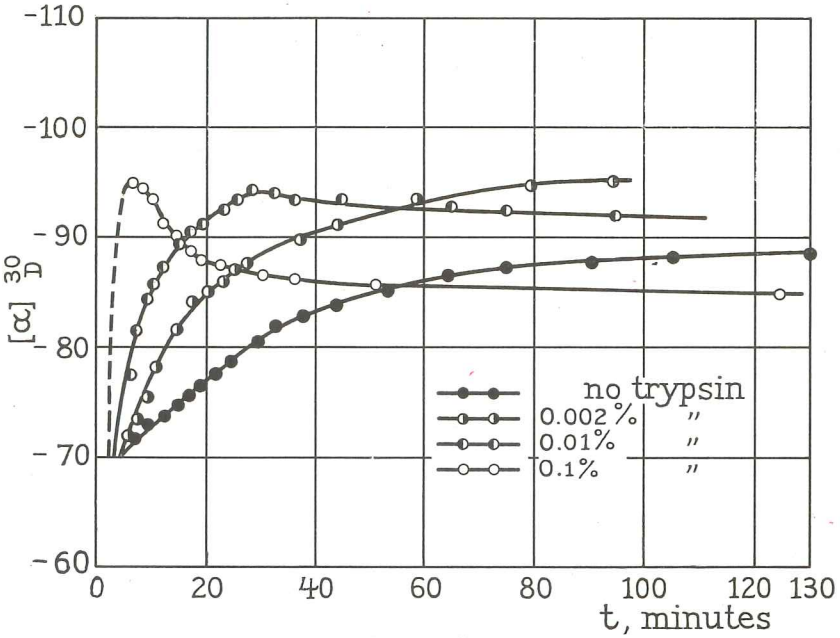


FIGURE 49

It is true that in the urea-containing systems denaturation is accentuated by this substance, but it must in general be considered unlikely that any agent including trypsin will act denaturing unless the protein molecule has a tendency, however slight, to denature beforehand. The different factors that favor denaturation may therefore work hand in hand (extreme acidity or alkalinity, temperature, denaturing agents). For this reason the whole question of the relationship between the enzymatic breakdown of proteins and their denaturation is a very interesting one, also, from a physiological point of view. In an unpublished paper Korsgaard Christensen has emphasized the general importance of denaturing agents in the process of proteolytic breakdown. For instance, the bile salts in the intestinal tract act as denaturing agents in very small concentrations, and it is likely that such factors are of importance in the digestion. I regret that time does not permit me to go more fully into this aspect of the question.

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IV. THE ENZYMATIC BREAKDOWN OF OVALBUMIN

In my last lecture I talked about several ways in which proteins could be broken down by proteolytic enzymes, and I mentioned some of the experiments carried out by Jacobsen on the activation of chymotrypsinogen by trypsin, in which a very slight chemical change of the attacked protein molecule was observed, causing a rather dramatic change in its behavior. The chymotrypsinogen molecule, which is inactive, becomes an enzyme capable of splitting proteins. Today, I shall tell another story about a relatively simple breakdown of a protein, namely, the breakdown of ovalbumin by a bacterial enzyme (1). The history of this investigation may be interesting because it started by our following the tradition of the Carlsberg Laboratory, introduced by Sørensen, never to throw anything away. We had in our refrigerator samples of ovalbumin which had been used for several purposes during a considerable time interval. And among these samples were some which had been applied in the study of "bound water" in ovalbumin, using urea or mannitol as the third component. After the experiments the ovalbumin samples were collected in a bottle and labeled "slops." Half a year later Dr. Ottesen, who has carried out most of the investigations that I am going to talk about today, began to work up, recrystallize, these slops. They didn't smell badly, probably because they were covered with kerosene which doesn't smell too good in a different way; but after inspection of the crystals he came to me and said, "It looks queer." We looked at the crystals together in the microscope and there, instead of the nice small needles that ovalbumin usually forms when it crystallizes, there were a number of rectangular plates instead, rather big ones. We decided that this phenomenon was worth investigation. At first we thought it was due to the urea or the mannitol, and we made a lot of negative experiments in which ovalbumin solutions containing urea or mannitol or both were placed in the refrigerator for different lengths of time, but finally we found that the best thing to do was just to let an ovalbumin solution stand at room temperature without toluene. In the course of a few days the transformation into this other protein which crystallized in plates had occurred, and at the same time a definite odor due to bacterial decomposition had developed. We rightly assumed that bacterial infection was the cause of our phenomenon. From then on, this problem became more scientific, if I may say so. We studied it by means of an enzyme obtained from *Bacillus subtilis*, the most common contaminating organism in the laboratory. We were fortunate enough to get a sample of this bacterial enzyme from Nordisk Insulin Laboratory in Copenhagen where scientists had been working with the isolation of bacterial enzymes. This enzyme, after a few purifications and precipitation with ammonium sulphate and dialysis, was able to do the job in the course of a few hours at a reasonably small concentration (about 0.1 percent) of the ovalbumin. The conversion of ovalbumin was