CCXCII. THE BEHAVIOUR OF PEPSIN IN THE ULTRACENTRIFUGE AFTER ALKALINE INACTIVATION.

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INTRODUCTION.

IN previous work [Philpot and Eriksson-Quensel, 1933] the behaviour of crystalline pepsin in the ultracentrifuge was shown to be that of a homogeneous substance of molecular weight about 35,000, with approximately spherical molecules. The work has now been extended to cover pepsin which has been inactivated by alkali.

The facts already known about alkaline inactivation are as follows [Northrop, 1930; Goulding *et al.*, 1927]:

(1) The change is very slow at p_H 6 and increases in speed with p_H until at p_H 9 it is practically instantaneous.

(2) Under the conditions used by Goulding *et al.* and by Northrop a definite fraction of the activity disappears instantaneously even at p_H 6, and this fraction increases with p_H .

(3) When the p_H is brought back to below 6, up to 10 % of the original activity can be restored. The optimum p_H for this reactivation is 5.4 [Northrop, 1931].

(4) Inactive pepsin is rapidly digested by active pepsin. This is probably one reason for the incompleteness of the reactivation.

The study of the changes in sedimentation constant accompanying the above changes is complicated by the fact that the solution in the ultracentrifuge is exposed for 1-3 hours to a temperature rising to 30-35° during the period of measurement. This makes it impracticable to distinguish "slow" from "instantaneous" inactivation at a p_H where both are supposed to occur; it is only possible to study the final result of the two processes and its dependence on p_H . It is also impracticable to study a solution containing a mixture of active and inactive pepsin at a p_H where digestion can occur. Further there is the problem of obtaining reproducible specimens of pepsin. Different ones used in this work had sedimentation constants varying from 2.9×10^{-13} to 3.7×10^{-13} . They must, therefore, have been slightly inhomogeneous, though this was not always apparent in the ultracentrifuge since with such small molecules slight inhomogeneity is obscured by diffusion. Also, although all the specimens were very well crystallised, the peptic activity as measured by the haemoglobin method varied between 60 and 80 % of the highest activity recorded by Northrop. Nevertheless it has been possible to observe certain definite phenomena which seem worth recording.

EXPERIMENTAL.

Ultracentrifuge. The work was done in Svedberg's laboratory at Upsala, using his oil-turbine ultracentrifuge [Svedberg, 1934], with the light absorption method of recording the sedimentation.

Preparation of pepsin. The crystalline pepsin was obtained by procedures slightly modified from that of Northrop [1930]. Each preparation had a slightly different history, as continual efforts were being made to improve the method. These intentional variations, together with unavoidable accidental ones, probably account for the variations in the final product which have already been mentioned. A perfect method of preparation has yet to be found, but the most promising one tried so far is as follows.

First crystallisation. Dissolve 100 g. Parke-Davis pepsin (1 : 10,000) in 100 ml. H_2O and add 100 ml. NH_2SO_4 . Filter through a large finest grade sintered Jena glass filter ("25G4") to remove traces of an insoluble slimy substance. Add 200 ml. saturated $MgSO_4$ through a capillary at a rate of 2 drops per second, using rapid mechanical stirring. (The slow regular addition gives a coarse granular precipitate which filters very easily.) Filter as above, removing as much mother-liquor as possible without letting the precipitate become dry. The moist precipitate weighs 50–70 g. according to the batch of starting material. Dissolve the precipitate, without washing, in a minimum volume of dilute acetate buffer (0.105 M CH_3COOH , 0.05 M $NaOH$, p_H 4.6). The volume required (ml.) is slightly less than the weight of precipitate (g.). Filter and leave to crystallise at room temperature, stirring mechanically at a rate just sufficient to prevent the crystals from settling out. Crystallisation is complete after 5–6 days: yield 10–18 g. moist crystals. The crystallisation can be hastened by using a higher temperature (e.g. 37°) but there is more decomposition. If the solution is not stirred it will deposit perfect crystals (about 1 mm. long) visible to the naked eye (some of these crystals were given to Bernal and Crowfoot [1934] for X-ray examination): but it will take a very long time to complete the crystallisation and the crystals will be stuck to the walls of the vessel.

Recrystallisation. Stir the moist crystals at room temperature for half an hour with acetate buffer of the same composition as above, but ten times as strong (i.e. 1.05 M CH_3COOH , 0.5 M $NaOH$), using a volume of buffer (ml.) equal to 1.2 times the weight of moist crystals (g.). Filter from the undissolved matter, which should be very little. Add slowly, with stirring, a volume of NH_2SO_4 equal to 0.45 times the volume of buffer taken. There should be no permanent precipitate: if there is the solution must be filtered and the residue discarded. Leave the clear solution to crystallise for 3 days at room temperature with slow mechanical stirring. Yield about 50%. Some of the pepsin used in the ultracentrifuge experiments had been recrystallised 2–3 times using the same dilute buffer as in the first crystallisation. The use of stronger buffer was introduced in order to obtain a pepsin concentration equal to that in the first crystallisation. Recrystallisation does not always increase the homogeneity or the specific activity and may even decrease it. The final product was sometimes dialysed against distilled water or dilute buffer to remove non-centrifugible matter.

p_H control. A typical experiment involved bringing the p_H from 4.6 to 9 and back to 4.6 again. In doing this a compromise was made between the following mutually antagonistic principles:

- (1) The solution used for changing the p_H must differ in p_H as little as possible from the final value for the mixture. This is to avoid local extremes of p_H during mixing with possible production of irreversible changes in the protein.

- (2) The buffer concentration in the final mixture must be low enough to avoid large corrections for viscosity and density in calculating the sedimentation constant, because the final concentration of each form of buffer cannot always be accurately estimated.

(3) At all stages the buffer concentration must be high enough for the p_H to be easily controllable.

In practice the p_H of the added buffer differed from the value after mixing by about 0.5 unit, and the mixing was done as rapidly as possible by pouring from one test-tube to another. The final total buffer concentrations were about 0.1 *M*. The measured p_H was usually within 0.3 of that calculated by approximate equations, whilst the correction for viscosity and density was from 1 to 6% of the sedimentation constant. The buffers used were acetate, phosphate and borate, according to the p_H . In calculating the quantity of buffer required to change the p_H , allowance was made for other buffers present in the solution, but not for the protein since its concentration was very low (about 0.1%). The p_H values recorded were obtained by measurement with a hydrogen or glass electrode, or were calculated, in which case the uncertainty probably does not exceed 0.3 unit.

RESULTS.

1. *The variation of sedimentation constant with p_H .* Pepsin solutions were brought to various p_H values and ultracentrifuged at once. The measured sedimentation constant corresponds therefore to the condition of the substance after 1–2 hours at this p_H . The results are shown in Fig. 1. The data are in-

Fig. 1. The variation of sedimentation constant of pepsin with p_H .

complete; but there is no doubt that in the region 7–11 the sedimentation constant falls with increasing p_H . At p_H 6–7, the region of "slow" inactivation, two different specimens gave quite different results. The curve has therefore been left undrawn in this region, as it requires much more detailed study. Even as

Fig. 2 a.

Fig. 2 b.

Fig. 2 c.

Fig. 2 d.

Fig. 2 e.

Fig. 2 f.

Fig. 2. Sedimentation diagrams of pepsin (for explanation see text). *a*, at p_H 11; *b*, at p_H 5; *c*, at p_H 4.6 after standing at p_H 6.7 for 10 min.; *d*, at p_H 4.6 after standing at p_H 6.7 overnight; *e*, at p_H 4.5 after standing at p_H 9 for 10 min.; *f*, at p_H 4.0 after standing at p_H 9 for 10 min.

far as p_H 11 the protein remains surprisingly homogeneous, as is shown by the sedimentation diagram in Fig. 2*a*. For comparison Fig. 2*b* shows pepsin at p_H 5.0 where no alkaline inactivation occurs. For contrast Fig. 2*c* shows a very inhomogeneous preparation which will be described later.

These sedimentation diagrams are obtained by photographing the solution in the ultracentrifuge with ultraviolet light at a series of instants from the start of sedimentation. The photographs are converted by means of a microphotometer into curves of which the abscissae are radial distances inside the ultracentrifuge cell and the ordinates are concentrations. The scales of ordinates and abscissae are arbitrary. If the protein is homogeneous there will be a sharp S-shaped portion with a nearly horizontal lower portion. Conversely a curve rising gently the whole way indicates inhomogeneity.

2. *The variation of sedimentation constant with final p_H of pepsin initially brought to p_H 9.* Pepsin was brought to p_H 9 and left for 10 min. at room temperature, which is sufficient for complete inactivation. It was then brought back to a p_H value ranging from 3.8 to 4.9. In Fig. 3 the sedimentation constant

Fig. 3. The variation of sedimentation constant with final p_H of pepsin initially brought to p_H 9. Points bracketed together represent components co-existing in solution.

is plotted against the final p_H . At final p_H 4.9 the sedimentation constant is slightly lower than that of normal pepsin. At final p_H 4.8 about 30% is transformed into molecules having a sedimentation constant nearly four times that of normal pepsin. At final p_H 4.7 the transformation is nearly complete. The sedimentation constant then rises with falling final p_H . There is pronounced inhomogeneity over the whole range, as is shown for instance in Fig. 2*e*, which

represents pepsin at a final p_H of 4.5. At final p_H 4.0 there are three distinct components, as is shown in Fig. 2*f*; their sedimentation constants are respectively about 10, 20 and 25 times that of normal pepsin. This is the last stage before precipitation, which is complete at final p_H 3.8.

3. *The variation of sedimentation constant with initial p_H of pepsin finally brought to p_H 4.5.* Pepsin was kept at p_H values between 6.7 and 11.6 for 10 min. and then brought to p_H 4.5. The results are shown in Fig. 4. Between initial

Fig. 4. The variation of sedimentation constant with initial p_H of pepsin finally brought to p_H 4.5. Points bracketed together represent components co-existing in solution. The point enclosed in a bracket was obtained on standing at the initial p_H overnight instead of for 10 min.

p_H 7.9 and 11 the final sedimentation constant is surprisingly constant, being about 10 times that of normal pepsin. As the initial p_H falls an increasing portion of the material suffers a fall instead of a rise in sedimentation constant and becomes very inhomogeneous. This portion is about 16% at initial p_H 7.9 and rises to 100% at initial p_H 6.7 even if the final p_H is as low as 3.8. However, if the pepsin is left at initial p_H 6.7 overnight the transformation to the higher sedimentation constant on bringing to p_H 4.5 is complete. Fig. 2*c* shows the effect of 10 min. at initial p_H 6.7. Not only is the mean sedimentation constant low, but the product is very inhomogeneous, probably through digestion of inactive by active pepsin. Fig. 2*d* shows the effect of 24 hours at initial p_H 6.7. The sedimentation constant is now much higher (the curve was obtained at a much lower rotational velocity).

DISCUSSION.

The most surprising thing about the results here described is the homogeneity of alkali-inactivated pepsin. The inactivation is regarded by Northrop as a case of alkaline denaturation, but this is usually accompanied by the production of marked inhomogeneity. It might be better to say that alkali converts pepsin into another protein which is more easily denatured by acid, since the substance formed on returning to p_H 4.5 is sufficiently inhomogeneous to be so regarded. The p_H at which this "acid denaturation" of alkaline pepsin begins, namely 4.8, is close to that found by Northrop [1931] to be the optimum for reactivation, namely 5.4. The optimum may therefore mean that reactivation can only proceed if the protein has not undergone "acid denaturation"; but it may merely be due to the more rapid digestion of inactive by active pepsin at lower p_H .

The changes in sedimentation constant admit of various interpretations. The slight fall on alkaline inactivation might be due to a change in molecular weight, in shape or in degree of hydration. The first might be detected by a determination of the sedimentation equilibrium. This has not yet been done. It should also be distinguishable through a rise in diffusion constant, whereas change in shape or increase in degree of hydration should cause a fall in diffusion constant. It has been found by Polsen (unpublished), in Svedberg's laboratory, that the diffusion constant falls. Thus the change is probably one of shape or degree of hydration. On the other hand the increase of sedimentation constant on "acid denaturation" of alkaline pepsin is so large that it must be due to aggregation. The fact that the sedimentation constant at a given final p_H is independent of initial p_H , as shown in Fig. 4, suggests that the p_H at which alkaline inactivation is performed has no fundamental influence on the nature of the final product.

SUMMARY.

1. When pepsin is brought to p_H 7 or higher the sedimentation constant falls, although the protein remains homogeneous.

2. When pepsin so treated is brought back to $p_H < 4.8$ a large rise in sedimentation constant occurs, and the protein becomes less homogeneous. The rise is greater the lower the final p_H , until at p_H 3.8 the protein is completely precipitated. The rise is approximately independent of initial p_H between p_H 6.7 and 11; but if the initial p_H is 6.7 it must be maintained for some time to be effective.

3. These results show that alkali-inactivated pepsin is distinguished by its homogeneity from "denatured" proteins; but that it has undergone some change which makes it more easily "denatured" by acid. They also suggest that the p_H at which alkaline inactivation is performed has no fundamental influence on the nature of the final product.

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