Pepsinogen and Pepsin: Conformational Relations, Studied by Iodination, Immunochemical Precipitation, and the Influence of Pepsin Inhibitor*

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Parameters which reflect conformational similarities and differences between purified pepsinogen and pepsin have been studied. Iodination of pepsinogen shows that, as in the case of pepsin, the residues essential for enzymatic activity are readily accessible. The data, in terms of target theory, indicate that 4–5 such residues are in the critical target area. Immunochemical precipitation studies by gel diffusion and quantitative precipitation also reveal conformational similarities but not identity for the two proteins. When both proteins are in a native state (*p*H 5.8) cross-reactivity is very strong. At *p*H 7.8, where pepsin is enzymatically inactive, the cross-reactivity is much weaker. Pepsin inhibitor was able to preserve the conformational integrity of the catalytic region in pepsin but was unable to restore either immunochemical identity with pepsinogen or even the strong cross-reaction seen at *p*H 5.8. In addition, pepsin inhibitor was able to restore activity to pepsin denatured at *p*H 7.8. This reactivation is postulated to occur by combination of the inhibitor peptide with the pepsin molecule before irreversible denaturation occurs.

Pepsinogen and pepsin are believed to be single-chain polypeptides (Van Vunakis and Herriott, 1957). The formation of pepsin from its enzymatically inactive precursor is accomplished by limited proteolysis. About 20% of the pepsinogen structure is removed from the *N*-terminal region of the protein, resulting in a reduction in molecular weight from about 42,500 to about 34,500 (Van Vunakis and Herriott, 1957). Details of conformational changes which may accompany this reduction in size are not known, nor is it known whether the active site of pepsin preexists in a shielded state in pepsinogen or is formed *de novo* by conformational changes during the conversion.

Changes in specific rotation of proteins at a given wave length indicate alterations in macromolecular conformation (Urnes and Doty, 1959). The small difference, 10° between the specific optical rotation values reported for pepsinogen, $[\alpha]_D^{pH\ 5.6}$ −62°, and −72° for pepsin (Herriott, 1938), suggests that conformational alterations may involve only limited portions of the molecule.[1] In terms of chemical properties, however, the two proteins exhibit marked differences in stability toward acid and alkali. Pepsin is stable in the acid *p*H range and pepsinogen in the neutral *p*H range (Northrop *et al.*, 1948).

The present study extends the characterization of pepsinogen and further defines the differences and similarities between the zymogen and pepsin relating to conformational changes and to the enzymatically active site. Iodination of pepsinogen and its effect on enzyme activity were studied, as were the reactions of pepsinogen and pepsin with antipepsinogen antibodies.

Multivalency of antigens makes possible the detection of structural changes other than those reflected by measurements of enzyme activity. Therefore, immunochemical studies were carried out at *p*H 5.8, where both proteins are stable, and also at *p*H 7.8, where pepsin is denatured. The influence of the pepsin inhibitor on the immunochemical and enzymatic properties of pepsin and on the reversal of inactivation of pepsin by alkali is also reported.

MATERIALS

Pepsin, Pepsinogen, Pepsin Inhibitor.[2] —The pepsins used were a three-times-crystallized preparation (Pentex Company, Inc., Lot No. D3709) and a two-times-crystallized preparation (Worthington Biochemicals Corp., Lot No. M628), obtained by fractionation with ethanol according to Northrop (1946).

Pepsinogen, obtained from Worthington Biochemicals Corporation (Lot No. 6007; 6007–10), was prepared by a modification of the method of Ryle (1960).[3] Lot 6007–10 contained about 2% insoluble material, which was removed before use. The proteolytic activity of the pepsinogen after conversion to pepsin and corrected for differences in molecular weight was about 23% greater than that of the crystalline pepsins when assayed by the hemoglobin method. This was confirmed by independent measurements by the manufacturer (private communication).

Pepsin inhibitor, estimated to be 80–90% pure, was obtained from Dr. Helen Van Vunakis, Brandeis University.

Rabbit Antiporcine Pepsinogen Serum. —Rabbits were immunized with 66 mg pepsinogen, administered intravenously in a 0.4% alum suspension. A series of 17 graded dose injections were given at the rate of three injections per week. Normal serum was obtained prior to the start of immunization. Antiserum was collected 1 week after the last injection.

In interpretation of the results of immunodiffusion

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[1] Calculations of the $[\alpha]_D$ from recent data of Perlmann and Harrington (1961) on pepsinogen and pepsin at 400 mμ using the modified Drude equation (Yang and Doty, 1957) yield values of −56° and −67°, respectively, for pepsinogen and pepsin, a difference of 11°. This confirms the small difference reported in the earlier work. The calculations utilize the fact (Jirgensons, 1958, 1959; Perlmann and Harrington, 1961) that optical rotatory dispersion of pepsin is not significantly affected by ionic strength, urea, and *p*H in the range 4.6–5.5. These facts were assumed to hold for pepsinogen in the *p*H range of its stability, *i.e.*, *p*H 7.7–5.5.

[2] Abbreviations that will be used are: Pg, pepsinogen; P, pepsin; Pi, pepsin inhibitor; P·Pi, pepsin–pepsin inhibitor complex or mixture.

[3] Egan, R., Worthington Biochemicals, Inc., private communication.

and immunoelectrophoresis experiments, normal serum and antiserum from only a single animal (#3113) was used in order to eliminate heterogeneity of antibodies of pooled sera. Furthermore, whole serum rather than globulin fractions was used to minimize the loss of antibody species that might be removed by fractionation.

Horse Antirabbit Globulin Serum.—The preparation of this antiserum has been described (Schlamowitz, 1958a).

Methods

Determination of Protein Concentration.—Protein concentration was determined by measurements of optical density at 280 mμ in the Beckman model DU spectrophotometer.

Determination of Proteolytic Activity of Pepsin and Pepsinogen.—Pepsin and pepsinogen (after activation) were assayed with hemoglobin (1.67%) as substrate. In all experiments except those in which antibody was present digestion was for 10 minutes at 37° at *p*H 2.7 in 3 ml total volume. When antibody was present *p*H 1.7 was used to assure complete dissociation of enzyme from antibody. Reaction mixtures were deproteinized with 5 ml of a 5% solution of trichloroacetic acid, and the absorbancy of the filtrates was measured at 280 mμ.

Pepsinogen was first activated at *p*H 1.7 for 15 minutes at about 25° before use. Activation was achieved in 1 minute or less at this *p*H.

Chromotography of Pepsinogen on DEAE-Cellulose.—Columns of DEAE-cellulose (0.9 × 20 cm) were charged with 10 mg pepsinogen. Elution chromatography of the zymogen was then carried out with the salt gradient of Liener (1960) at a flow rate of about 12.8 ml per hour.

The DEAE-cellulose was preequilibrated with 0.1 M phosphate buffer, *p*H 6.8, and packed under 2.5 p.s.i. nitrogen. All operations were carried out at 5°.

Ultracentrifugal Analysis of Pepsinogen.—Sedimentation studies were carried out at 20° in a Spinco analytical ultracentrifuge, model E, at 59,780 rpm with the standard analytical cell. Diffusion measurements were made with the synthetic boundary cell at 12,590 rpm. Pepsinogen solutions were preequilibrated by dialysis against 0.15 M NaCl, 0.0015 M phosphate, *p*H 7.0, ionic strength 0.153.

Values of the sedimentation coefficient were obtained from the slope of the line relating the logarithm of the boundary position as a function of time (Schachman, 1959) and corrected to water at 20° ($S_{20,w}$). The diffusion coefficient was obtained from the slope of the line obtained by plotting (area)^2/(maximum height)^2 *versus* time, *i.e.*, the "maximum ordinate-area method" (Ehrenberg, 1957), and corrected to water at 20° ($D_{20,w}$). Molecular weights were calculated from the equation, $M = RTs/D(1 - \bar{v}\rho)$. The value of 0.750 ml per g for the partial specific volume, $\bar{v}$, of pepsinogen was calculated from the amino acid analysis of pepsinogen (Van Vunakis and Herriott, 1957) and the partial specific molar residue volumes of amino acid residues in proteins (Cohn and Edsall, 1948). A value of 1.006 g per ml was used for the density, ρ, of the solution (Svedberg and Pedersen, 1940).

Electrophoresis.—Samples were examined by free boundary electrophoresis in 0.1 M phosphate buffer at *p*H 6.8 in the Perkin-Elmer electrophoresis apparatus. The pepsinogen (0.553%) was equilibrated by dialysis overnight against the buffer.

Electrophoresis on cellulose-acetate was carried out in the Shandon apparatus (Consolidated Labs. Inc., Chicago Heights, Ill.) at room temperature in 0.05 M phosphate buffer at *p*H 6.8. The time of electrophoresis was 3.5 hours and the current 0.4 milliamp per cm width of strip. Nigrosin (1% in 2% acetic acid) or Ponceau S was used to locate the protein bands.

Immunodiffusion. — Immunodiffusion (2% Noble-Difco agar in appropriate phosphate buffer, 3 mm thick) was carried out at room temperature. The wells (1 mm diameter placed 5 mm apart) were filled with about 9.4 μl of solution, and diffusion was allowed to proceed in a moist chamber for about 20 hours. Precipitation bands were photographed either directly or after staining with nigrosin.

Phosphate buffers, 0.004 M, *p*H 5.8 and 7.8, of high ionic strength (0.2 M NaCl) and low ionic strength (no added salt), were used for making up the agar as well as for preparing solutions of pepsin, pepsinogen, and pepsin inhibitor. Serum samples were also adjusted to *p*H 5.8 or 7.8 before use.

Immunoelectrophoresis.—Electrophoresis of normal and immune serum was carried out at room temperature in agar (2 mm thick) in the Agafor-Egton AG apparatus at *p*H 5.8 (0.04 M phosphate buffer) for 1 hour. The applied voltage across the agar was 30 volts. Electrophoresis at *p*H 8.2 (0.05 M Veronal buffer) was carried out under similar conditions but for 1.25 hour. The lateral troughs were then filled with pepsin or pepsinogen in the same buffer or with horse antirabbit globulin serum, and diffusion was allowed to proceed for about 20 hours. Precipitation bands were photographed as described above.

Iodination of Pepsinogen and Determination of Radioactivity.—I^{131}-labeled hypoiodite was prepared in glycine buffer (Pressman and Roholt, 1961) and diluted with 1 N NaCl to give about 1.6 × 10^6 cpm per ml. For iodination, solutions containing 0.1 ml of labeled hypoiodite, 0.32 ml of unlabeled ICl (0.24–7.68 μmoles in 1 N NaCl), and 1.58 ml of 0.2 M borate buffer, *p*H 8.2, for a total volume of 2.0 ml, were added to equal volumes of pepsinogen solutions, each containing 5.1 mg pepsinogen (0.12 μmole, molecular weight 42,500; Van Vunakis and Herriott, 1957) in 0.001 M phosphate, 0.10 M borate buffer, *p*H 8.2. Iodination was allowed to proceed for 0.5 hour in the cold. Reaction mixtures were then dialyzed in the cold, first against several changes of 0.02 M borate, *p*H 8.2, containing 8 g NaCl and 30 mg KI per liter, and then against 0.1 M phosphate, *p*H 6.8, until the dialysates showed no radioactivity above background. All solutions remained clear. Portions of the dialyzed solutions were then analyzed for protein, radioactivity, and proteolytic activity.

Protein content was assayed by comparing the absorbancy at 280 mμ, the isosbestic point of tyrosine and diiodotyrosine (Beaven and Holiday, 1952), with the absorbancy of known solutions of pepsinogen.

Radioactivity was assayed in a well-type scintillation counter and corrected for background. The counting rates of 4000–8500 cpm per ml were below the limits for coincidence errors, and counting periods of 1 or 2 minutes, depending on rate, were used. The number of iodine atoms introduced per mole of pepsinogen was calculated from the specific radioactivity of these samples, corrected for decay of the I^{131}.

Suitable dilutions of control and iodinated solutions of pepsinogen were activated and assayed for proteolytic activity as described above.

Constant-Antigen Precipitation Reactions of Pepsinogen and Pepsin.—These reactions were carried out essentially as previously described (Schlamowitz, 1958b). Constant amounts of the test antigens, pepsinogen or pepsin, at *p*H 5.8 or 7.8, were added to a

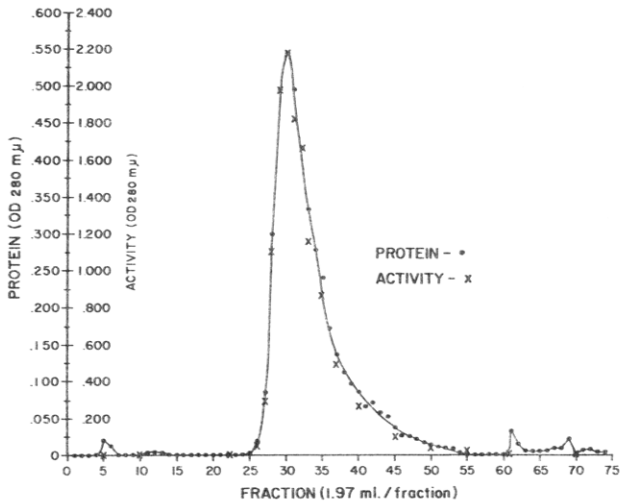


FIG. 1.—Chromatography of pepsinogen on DEAE-cellulose. See text for details.

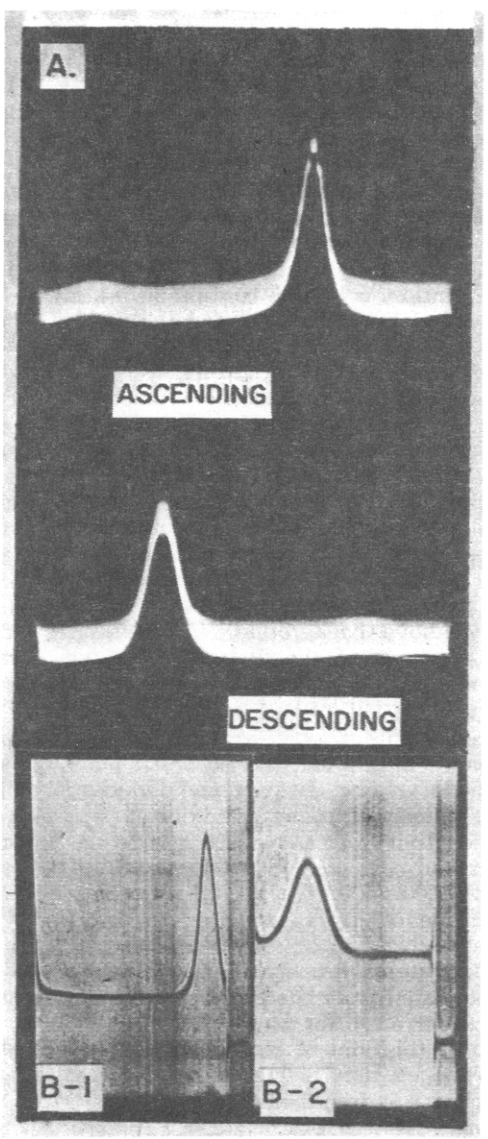


FIG. 2.—Electrophoresis and sedimentation of pepsinogen. *A*, free boundary electrophoresis, 5700 sec. *B*-1, sedimentation pattern after 20.6 min. *B*-2, sedimentation pattern after 164.7 min.

series of tubes containing increasing volumes of anti-pepsinogen serum adjusted to the same *p*H. Normal serum was added to maintain a constant serum volume. The mixtures were incubated for 5 minutes at 37°, for 1 hour at 25°, and then overnight at 5° before being centrifuged at 2000 × *g* at 5°. Portions of the supernatant fluids were assayed for proteolytic activity as described above, but with the substrate at *p*H 1.7 (*cf.* Methods, Determination of Proteolytic Activity). Under these conditions pepsinogen is activated almost instantaneously. Analysis of these supernatants for the amount of antigen precipitated agreed with values obtained by direct activity analysis of washed precipitates.

The antibody content of the precipitates was obtained from absorbancy at 280 mμ of solutions of the well-washed precipitates in 0.5 M acetic acid, corrected for absorbancy of antigen in the precipitate.

EXPERIMENTAL AND RESULTS

A. Characterization of Pepsinogen

The chromatographic, ultracentrifugal, and electrophoretic properties of pepsinogen were investigated to assess its physicochemical state of purity before it was used as an antigen in the immunochemical phases of this study.

Chromatography.—Elution chromatography of pepsinogen was carried out as described and the eluates were analyzed for protein and enzyme activity. The recovery of protein was better than 95%, and essentially all of the recovered material appeared under one peak (Fig. 1). The activity data showed that all the protein in this peak was associated with enzyme material.

Free Boundary Electrophoresis and Ultracentrifugation.—Pepsinogen migrated as a single peak in free boundary electrophoresis at *p*H 6.8 and in the ultracentrifuge at *p*H 7.0 (Fig. 2*A*,*B*).

The $S^{1\%}_{20,w}$ value was 3.28 *S*. Diffusion measurements yielded a value for the diffusion coefficient, $D^{1\%}_{20,w}$, of 7.34×10^{-7} cm²/sec. The molecular weight of pepsinogen calculated from these data was 43,500—in good agreement with 42,860 obtained from amino acid analysis, 42,500 from phosphorus analysis and osmotic pressure measurements (Van Vunakis and Herriott, 1957), and 41,000 obtained from sedimentation studies.[4]

As with many proteins, pepsinogen showed a linear concentration dependence of the sedimentation coefficient. From measurements at 0.25, 0.50, and 1.0% pepsinogen concentrations it was found that the dependence could be expressed by the equation $S_{20,w} = 3.50\ [1 - 0.06\ (c)]$, where c is the concentration of protein in g/100 ml, and 3.50 is the value of $S^{\circ}_{20,w}$. The magnitude of the change in $S_{20,w}$ with concentration is about the same as reported for serum albumin (Ehrenberg, 1957).

Assuming a model of a prolate ellipsoid of revolution (Wyman and Ingalls, 1943), the f/f_0 value for pepsinogen is 1.25 and the axial ratio for a nonhydrated ellipsoid is about 5:1. Pepsin, with an f/f_0 value of 1.08 (Greenberg, 1951; Green and Neurath, 1954) and a calculated axial ratio of 2.6:1, is a more symmetrical structure than pepsinogen.

Electrophoresis on Cellulose-Acetate.—Pepsinogen was found to contain a major component, *A*, and two minor components, *B* and *C* (Fig. 3). Component *C* migrates like denatured pepsin. Components *B* and *C* together make up about 2.5% of the total protein as judged by

[4] Perlmann, G. E., private communication.

comparison with known amounts of pepsinogen and pepsin. Such minor components would not be expected to be revealed by free boundary electrophoresis or by ultracentrifugal studies.

On the basis of the chromatographic, sedimentation, and electrophoretic criteria, the pepsinogen was judged to be at least 97.5% homogeneous. At this level of purity it was considered suitable for use in the chemical and immunochemical studies described below.

B. Iodination of Pepsinogen

Pepsinogen activity declined without a lag during iodination, and a semilogarithmic plot of the decline as a function of the number of iodine atoms introduced per molecule was linear (exponential survival) for at least 87% of the process (Fig. 4).[5] In these respects it behaved like pepsin (Herriott, 1937, 1954, 1956), in that iodination affected readily iodinated amino acid residues whose integrity is essential for catalytic function.

The number of such residues may be deduced from the survival data by use of target theory if the pepsinogen molecules are viewed as molecular targets for the iodine. However, while exponential behavior is consistent with the kinetics of target theory, it does not *per se* prove the operation of this mechanism.

When applied to functionally active molecules, simple target theory supposes a population of target molecules, each of which contains a number of structures which are sensitive to the inactivating agent used. It predicts that a hit made on any one of these structures will result in the loss of activity of the molecule, *i.e.*, one hit–one kill, even though the remaining sensitive structures in a target are still subject to subsequent hits. In a population of such target molecules hit randomly, the fraction which survives, c/C_0, is exponentially related to D, the dosage of inactivating agent absorbed, and is given by the equation $c/C_0 = e^{-D}$. When D equals 1, the surviving fraction of target molecules is 0.37 (Hutchinson and Pollard, 1961).

For pepsinogen specifically, the following assumptions will be made: (*a*) pepsinogen has one potential catalytic site (active after conversion to pepsin); (*b*) tyrosines are the sole amino acid residues iodinated,[6] *i.e.*, the sensitive residues; (*c*) of 16 tyrosines in pepsinogen[4] and pepsin, 10 are iodinated rapidly and at about the same rate,[7] and will be referred to as the "total target area"; (*d*) diiodotyrosine is formed preferentially during iodination of tyrosine,[8] so that the number of tyrosine residues substituted will be considered equal to half the number of iodine atoms taken up.

In the case of pepsinogen, if each sensitive tyrosine residue were essential, the iodination of any one would result in complete loss of activity of the molecule. The 37% survival value (1 dose) would then have occurred when 1 tyrosine (2 iodine atoms) was substituted per molecule. The experimentally determined value for 1 dose, however, was 2.3 tyrosines (4.6 iodine atoms per molecule, Fig. 4).

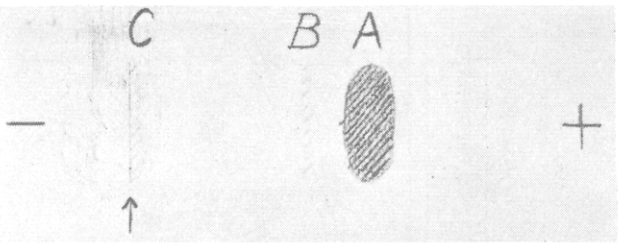


FIG. 3.—Cellulose-acetate electrophoresis of pepsinogen. See text for details.

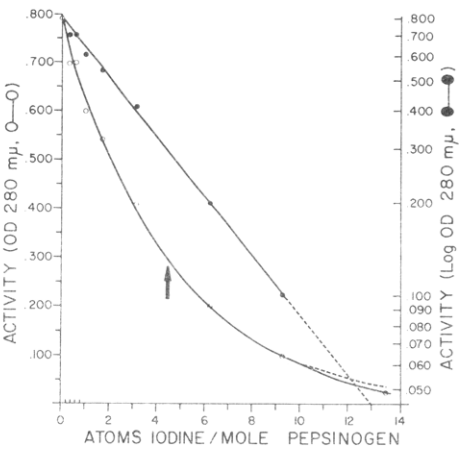


FIG. 4.—Loss of activity of pepsinogen by iodination. See text for details.

Since the linear semilog plot conforms to kinetics of a one hit–one kill mechanism rather than a multihit mechanism,[9] it is deduced that only hits on sensitive tyrosines in *part* of the "total target area" can accomplish kills. Thus, within the "total target area" there is a "critical target area"; iodination of any one of its tyrosines will cause complete loss in activity of the molecule. Under these conditions, kinetics for a one hit–one kill mechanism will still apply, but since only those hits in the "critical area" accomplish kills, the value for 1 dose will be found to be greater than 1 tyrosine (2 iodine atoms) per molecule. The "size" (number of sensitive tyrosines) of the "critical target area" is calculated from the dosage value. Thus, if as in the case of pepsinogen 2.3 tyrosines of the "total target area" were iodinated to ensure that one be in the "critical target area," the "critical target area" is 1/2.3 of the "total target area." In terms of the number of sensitive tyrosines this is 4.3 tyrosines, *i.e.*, $1/2.3 \times 10$.

Enzymatic integrity of pepsinogen appears then to depend on the conformational integrity of only a *portion* of the molecule, containing no more than 4–5 readily iodinatable tyrosine residues.[10]

[5] Deviation from linearity beyond this point may be due to experimental error at low enzyme concentrations.

[6] Herriott (1947) found that virtually all the iodine in pepsin could be accounted for as substituted tyrosine.

[7] Except when pepsin is completely denatured, all of its tyrosine groups are not available for iodination (Li, 1945). Based on a molecular weight for pepsin of 34,500, the experimental data of Li can be fitted to the second-order kinetics postulated by him for the iodination of tyrosine residues to diiodotyrosine, when the number of easily iodinated tyrosine residues is taken as 10. The semilogarithmic plot of the survival data for pepsinogen also extrapolates to a value of about 10 tyrosine residues at 99% inactivation.

[8] This assumption appears valid in light of the agreement of the kinetics of iodination of albumin, pepsin, and tyrosine with pseudobimolecular kinetics (Li, 1942, 1945) for the extent of iodination studied.

[9] Mechanisms which involve two or more hits in the target to achieve a kill (multihit survival curve) do not give linear semilogarithmic survival curves, nor do situations involving two exponentials (Hutchinson and Pollard, 1961).

[10] A critical target area containing just a single iodinatable tyrosine residue may be ruled out. The application of target theory to such a system would give rise to a linear survival curve given by the equation $c = C_0 - (C_0/M_0) \cdot Y$ rather than the observed exponential curve. C and C_0 have the same meaning as already given in the text. M_0 is the total concentration of iodinatable residues per target molecule, and Y is the number of such groups substituted.

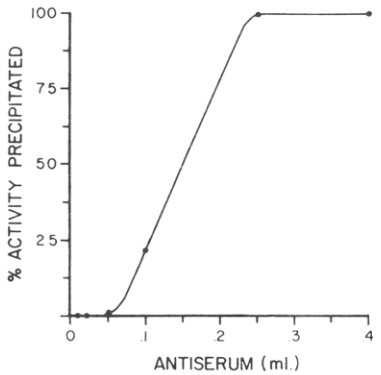


FIG. 5.—Precipitation of pepsinogen (100 μg) by rabbit antipepsinogen serum at *p*H 7.8.

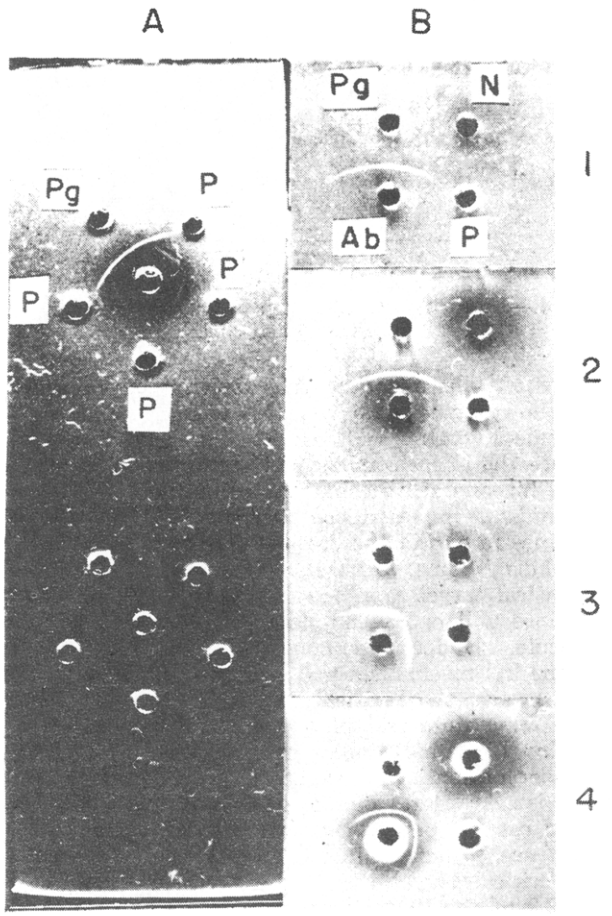


FIG. 6.—Immunodiffusion of pepsinogen and pepsin. *A*, Diffusion of pepsinogen, Pg (300 μg/ml) and pepsin, P (260, 195, 130, 65 μg/ml; clockwise) against antiserum in center well, top; and normal serum, bottom. *B*, Diffusion of pepsinogen, Pg (350 μg/ml) and pepsin, P (275 μg/ml) versus normal serum, N, and antipepsinogen serum, Ab. B-1, *p*H 7.8 high ionic strength; B-2, *p*H 7.8 low ionic strength; B-3, *p*H 5.8 high ionic strength; B-4, *p*H 5.8 low ionic strength. See text for details.

C. Immunochemical Properties of Pepsinogen, Pepsin, and Pepsin Inhibitor

Titration of Pepsinogen with Antipepsinogen Serum at pH 7.8.—A solution of pepsinogen (0.20 ml) containing 100 μg protein in 0.004 M phosphate buffer, 0.2 M NaCl, *p*H 7.8, was mixed with antiserum (0.01 – 0.40 ml) and normal serum at this same *p*H. The total

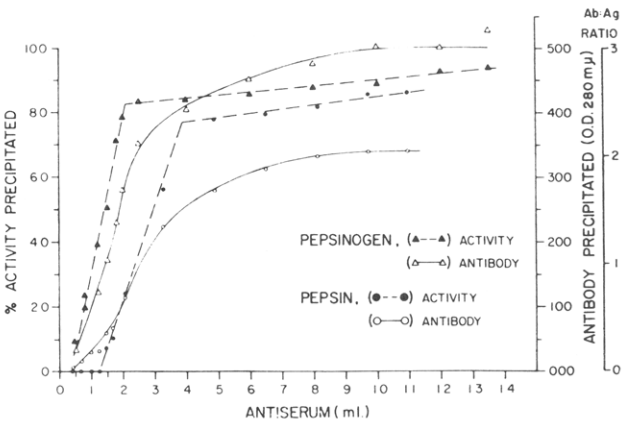


FIG. 7.—Precipitation of pepsinogen and pepsin by antipepsinogen serum at *p*H 5.8. See text for details.

volume of serum (immune and normal) was 0.60 ml. The mixtures were incubated and centrifuged at 18,000 × *g*, and portions of the supernatant fluids were analyzed for pepsinogen as described.

Figure 5 shows that pepsinogen was completely precipitated by its antiserum. About 0.25 ml of antiserum was required for complete precipitation of 100 μg of pepsinogen. At levels of antiserum below 0.05 ml, in the region of antigen-excess the enzyme was not precipitated at all.

Immunodiffusion of Pepsinogen and Pepsin.—Pepsinogen (300 μg per ml) gave a single band with its antiserum at *p*H 7.8, low ionic strength. Under these conditions pepsin (65–260 μg per ml) showed weak lines of immunochemical partial-identity, though its enzymatic activity was destroyed. Neither substance reacted with normal serum (Fig. 6*A*). Destruction of enzymatic activity therefore is not accompanied by complete elimination of the cross-reacting antigenic determinant conformations. Partial cross-reaction of pepsin with antipepsinogen serum at about neutral *p*H has also been reported recently by Freedberg *et al.* (1962).

As shown in parts 1 and 2 of Figure 6*B*, the same reactions are observed at both low and high ionic strength.

At *p*H 5.8, where control studies showed pepsinogen and pepsin to be enzymatically stable, the immunochemical reaction with antipepsinogen serum was strong for both substances, again at both low and high ionic strengths (parts 3 and 4 of Fig. 6*B*). At this *p*H, however, the spur on the pepsinogen line was very weak. This fact suggests that only a small number of determinant groups is lost in the zymogen-to-enzyme conversion and that the difference in the capacity of the two to accommodate antibodies on their surfaces is not great.

This last point was confirmed in quantitative constant-antigen precipitation studies of pepsinogen and pepsin at *p*H 5.8 with antipepsinogen serum (Fig. 7). Antibody-antigen ratios of 3:1 and 2:1, respectively, for pepsinogen and pepsin were calculated from the extrapolated values for antibody protein in the washed precipitates (corrected for antigen) formed in excess antibody. In these calculations, 160,000 was taken for the molecular weight of antibody and 14.9 for its specific absorbancy at 280 mμ (Gitlin, 1949). If it may be assumed that in excess antibody the minimal requirement for precipitation is a linear copolymer of antigen and antibody, the immunochemical valences of pepsinogen and pepsin would be 4 and 3 respectively.

In any event the data show that conversion of pep-

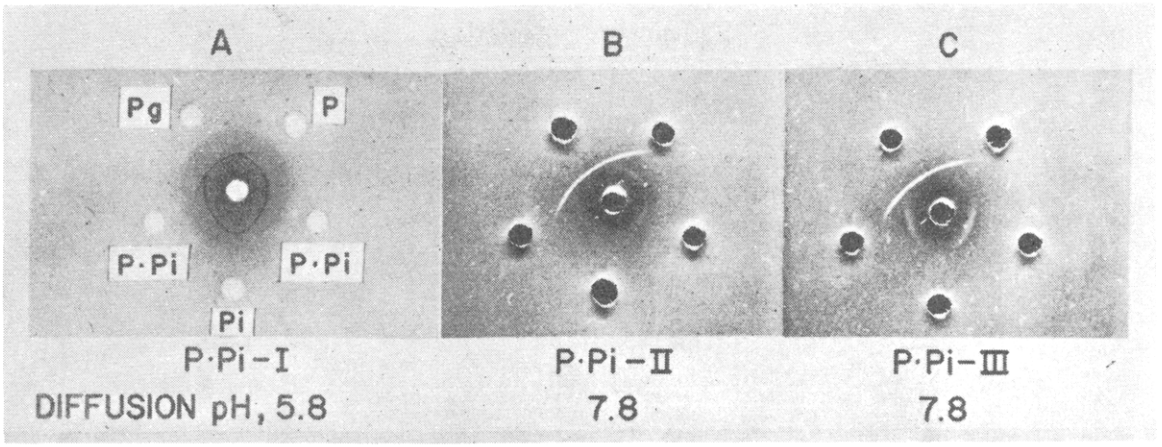


FIG. 8.—Immunodiffusion of pepsinogen, Pg (300 μg/ml), pepsin, P (243 μg/ml), pepsin inhibitor, Pi (105 μg/ml), and pepsin–pepsin inhibitor mixtures, P·Pi (243 μg P, 105 μg Pi/ml.). See text for details of preparation of P·Pi mixtures I, II, III.

sinogen to pepsin is associated with the alteration of only a limited portion of the molecule.

Effect of Pepsin Inhibitor on the Immunodiffusion Properties of Pepsin.—A comparison of the immunodiffusion characteristics of pepsinogen, pepsin, and pepsin plus pepsin inhibitor was made to determine whether combination of pepsin with the inhibitor would change the immunochemical properties of pepsin to those of pepsinogen.

Solutions of pepsin (P) and the inhibitor (Pi) were mixed, and the immunodiffusion against antipepsinogen serum was studied under three sets of conditions (Fig. 8). With P·Pi-I the solutions were mixed and diffused at *p*H 5.8, conditions under which pepsin is native and known to combine with the inhibitor (Northrop *et al.*, 1948) and pepsinogen is still native. With P·Pi-II the solutions were mixed and diffused at *p*H 7.8. Pepsin is enzymatically inactivated at this *p*H. With P·Pi-III the solutions were prepared and mixed at *p*H 5.8 and then adjusted 15 minutes later to *p*H 7.8 for diffusion. The mole ratio of pepsin inhibitor–pepsin used was greater than 3:1. At least 90% of pepsin is complexed with inhibitor under these conditions at *p*H 5.8 (Herriott, 1941). Equimolar amounts of pepsinogen and free pepsin and inhibitor were also diffused at these same *p*H values.

At *p*H 5.8, pepsin plus inhibitor, P·Pi-I, yielded a complex which was immunochemically indistinguishable from pepsin but clearly not identical with pepsinogen (Fig. 8*A*). The inhibitor alone gave no precipitation line with the antiserum. Thus the determinant groups lost by conversion of pepsinogen to pepsin are not present in the pepsin inhibitor nor are they restored by combination of the enzyme with inhibitor. As will be shown in section *D*, stabilization of enzyme activity toward alkali denaturation is conferred on pepsin by the inhibitor.

With both P·Pi-II and P·Pi-III (Fig. 8*B*,*C*) weak partial-identity lines were observed as for pepsin itself, but perhaps somewhat stronger. Stabilization of the catalytic region of pepsin by inhibitor in P·Pi-III (see section *D*, below) was apparently unable to confer stability on the other immunochemical determinants of pepsin against alkali denaturation.

It is possible that the small fraction of activated pepsinogen which Seastone and Herriott (1937) reported to react with some antipepsinogen antibodies in antipepsinogen serum was pepsin-pepsin inhibitor complex.

Immunoelectrophoresis of Antipepsinogen Serum.—To determine whether the antibodies were associated with more than one electrophoretic component, the rabbit antiserum was subjected to electrophoresis in agar at *p*H 5.8 and at *p*H 8.2. Immunodiffusion with pepsinogen at these *p*H levels was then carried out. In both cases only a single precipitation arc was seen (Fig. 9*A*,*B*). The antibodies cross-reacting with pepsin are in the same electrophoretic fraction (Fig. 9*C*). Normal serum did not react with either substance under these conditions.

A comparison of the pepsinogen arc with the characteristic long precipitation band given by the reaction of rabbit serum γ-globulins with horse antirabbit globulin serum shows that the antipepsinogen antibodies are in one of the slow-moving γ-globulin fractions (Fig. 9*A*,*B*).

D. Effects of Pepsin Inhibitor on the Enzymatic Properties of Pepsin

Enzymatic Activity of Pepsin-Pepsin Inhibitor Mixtures.—A comparison was made of the enzymatic activity at *p*H 2.7 of pepsin samples prepared at *p*H 4.5 (reference sample) and at *p*H 5.8 and 7.8 and of pepsin-pepsin inhibitor mixtures prepared at *p*H 5.8 and 7.8, and at *p*H 5.8 followed by adjustment to *p*H 7.8 (Table I). The data show that only the last mixture, P·Pi-III, had the *p*H stability characteristics of pepsinogen, although it immunochemically resembled both pepsin denatured at *p*H 7.8 and the inactive P·Pi-II mixture (*cf.* section *C*). It is apparent that prior formation of pepsin–pepsin inhibitor complex

TABLE I

PROTEOLYTIC ACTIVITY OF SOLUTIONS OF PEPSIN (P) AND MIXTURES OF PEPSIN PLUS PEPSIN INHIBITOR (P·Pi) PREPARED AT DIFFERENT *p*H VALUES

The activities of all preparations were measured at *p*H 2.7 with hemoglobin substrate. See text for details.

Enzyme	*p*H[a]	Relative Activity
P	4.5	100
P-I	5.8	99
P-II	7.8	0
P·Pi-I	5.8	100
P·Pi-II	7.8	14
P·Pi-III	5.8 → 7.8	97

[a]The *p*H values at which solutions of the enzyme or enzyme plus inhibitor were prepared. In the case of P·Pi-III the mixture was first mixed at *p*H 5.8 and subsequently adjusted to *p*H 7.8.

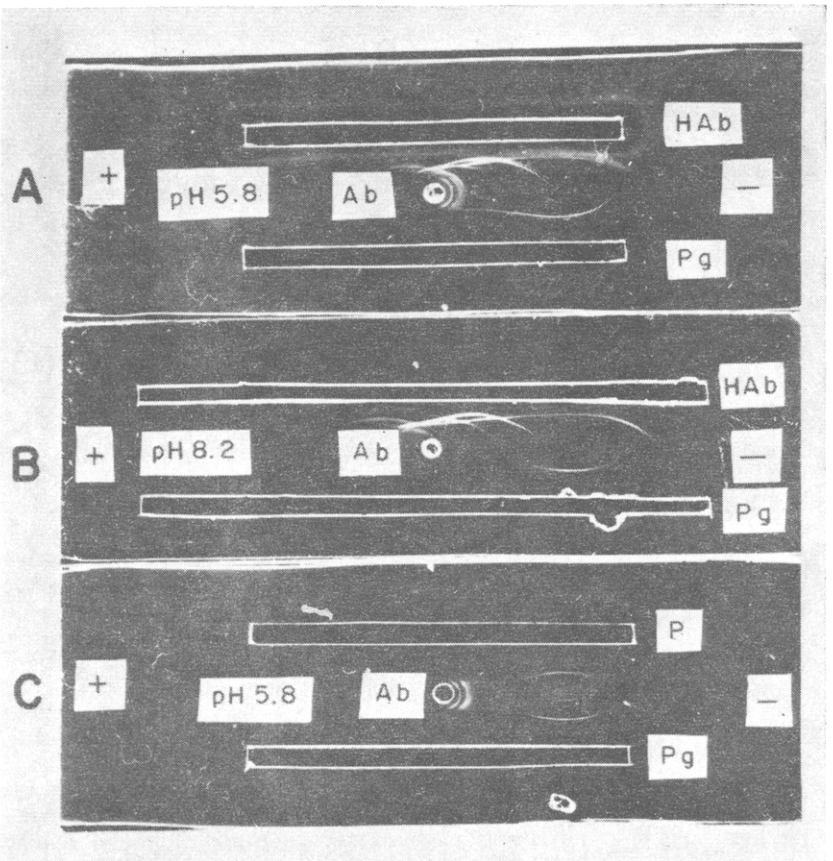


FIG. 9.—Immunoelectrophoresis of antipepsinogen serum, Ab, followed by: *A*, immunodiffusion with pepsinogen, Pg, and horse antirabbit globulin, HAb, at *p*H 5.8; *B*, same as for *A* but at *p*H 8.2; *C*, electrophoresis at *p*H 5.8 and diffusion with pepsinogen, Pg, and pepsin, P, at *p*H 5.8.

conserves the structure in pepsin that is essential for enzyme activity and that this structure does not depend on conservation of all of the immunochemical sites of native pepsin.

Effect of Pepsin Inhibitor on Reversible Denaturation of Pepsin.—The real (14%), albeit low activity of P·Pi-II implied either that 14% of the pepsin was not yet inactivated at *p*H 7.8 when the inhibitor was mixed with it (about 10 minutes after preparation of the *p*H 7.8 pepsin solution) or that the alkali-treated pepsin was partially renatured.

To test these alternatives, solutions of pepsin were prepared at *p*H 7.8 and aliquots were withdrawn for activity assay after varying periods of time (5, 18, 35, and 95 minutes and 24 hours) (Fig. 10, control curve). Other aliquots were simultaneously withdrawn at these times (shown by points *A*, *B*, *C*, and *E* on control curve) and mixed with about 3 M equivalents of a *p*H 7.8 pepsin inhibitor solution, and these mixtures were in turn analyzed for activity (Fig. 10-*A'*, *B'*, *C'*, *E'*) immediately after mixing and also 15, 30, and 60 minutes after mixing.

Pepsin alone at *p*H 7.8 showed a precipitous fall in activity as a function of time (Fig. 10, control curve). Only about 2.4% of the initial activity remained after 5 minutes (point *A*). With longer exposure practically all activity was destroyed (points *B*, *C*, *D*, *E*). In contrast, a sample withdrawn after 5 minutes (point A) and mixed with inhibitor showed 30% of the initial activity and retained over 22% even 1 hour after mixing (Fig. 10, *A'*).

After exposure of pepsin at *p*H 7.8 for 18 and 35 minutes (points *B*, *C*), 5–6% of the activity could still be restored by the inhibitor (Fig. 10, *B'*, *C'*). Data for restoration of activity after 24 hours at *p*H 7.8 are too meager to be interpreted at present.

DISCUSSION

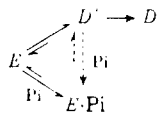
Although the amino acid composition of pepsinogen and pepsin is known (Van Vunakis and Herriott, 1957; Blumenfeld and Perlmann, 1959), as is the composition of one of the fragments produced in the conversion, *i.e.*, pepsin inhibitor (Van Vunakis and Herriott, 1956), little is known of the conformational changes which attend the transformation except that the difference in specific optical rotation values for the enzyme and the zymogen is small (*cf.* introduction). Since about 20% of the pepsinogen structure is eliminated during conversion, it is not surprising that some alteration in molecular conformation occurs, but whether the conformational alteration is secondary to the unmasking of a preexisting active site in pepsinogen or whether it reflects *de novo* formation of such a site is not known. The ease with which groups essential for the activity are iodinated can at best only suggest that they are present in exposed parts of the molecule that fold to form a site *de novo*. However, as with ribonuclease (Singer and Richards, 1959), it is felt that a definitive choice between these alternatives cannot be made at this time. For the trypsinogen and chymotrypsinogen systems changes in primary structure are quite small and the kinetics of change in optical rotation during conversion have been interpreted to favor *de novo* formation of a site (Neurath and Dixon, 1957; Imahori *et al.*, 1960).

Regardless of whether preexistence or *de novo* forma-

tion of a site is involved, the small difference in the values of specific optical rotation of pepsinogen and pepsin suggests that large segments of the pepsinogen structure are not involved in the conversion. The immunochemical results of the present study bear this out but point out some of the structural differences as well. Thus, at *p*H 5.8, where both proteins are stable, the immunochemical cross-reactivity, seen in both gel diffusion (Fig. 6*B*, parts 3, 4) and in quantitative precipitation (Fig. 7) studies, is considerable. From the antibody-antigen ratios of 3:1 and 2:1 it is estimated that pepsinogen and pepsin, respectively, can accommodate about 4 and 3 antibody molecules per molecule. Conformational similarities of pepsin and pepsinogen are reduced at *p*H 7.8, where pepsin also loses its catalytic activity (compare Fig. 6*B*, parts 1, 2, with 6*B*, parts 3, 4).

The immunological identity-patterns for pepsin and the pepsin-pepsin inhibitor complex at *p*H 5.8 (Fig. 8*A*) show that combination with inhibitor does not restore to pepsin the determinant groups that existed in pepsinogen. Although the inhibitor was able to preserve the integrity of the catalytic area when the enzyme was subsequently exposed to the denaturing effects of *p*H 7.8 (Table I), it was unable to protect against denaturation and loss of many of the cross-reacting determinant groups; *e.g.*, note similarity of precipitin characteristics of Figure 8*B* and *C*.

A mechanism to account for the restoration of pepsin activity at *p*H 7.8 (Fig. 10) may be depicted schematically. It is predicated on the denaturation of pepsin being a multistage process, one of the steps being reversible.



E is the enzyme region whose integrity is essential for activity, D' is an inactive but reversibly denatured form of this region of the enzyme, and D an irreversibly denatured, inactive form. At any particular time after exposure to *p*H 7.8 the enzyme would be distributed in some characteristic manner among these forms. The action of inhibitor, Pi, would then be to trap E in the form of the complex $E \cdot Pi$ and thereby shift the equilibrium $E \rightleftharpoons D'$ until all of both forms, E and D', was trapped as $E \cdot Pi$. Direct combination of Pi with D' as a way of trapping E and D' in an active state is also conceivable.

The region in pepsinogen essential for its potential pepsin activity was estimated to contain 4–5 readily iodinatable tyrosine residues, on the basis of a model in which pepsinogen is the molecular target for the iodinating agent.

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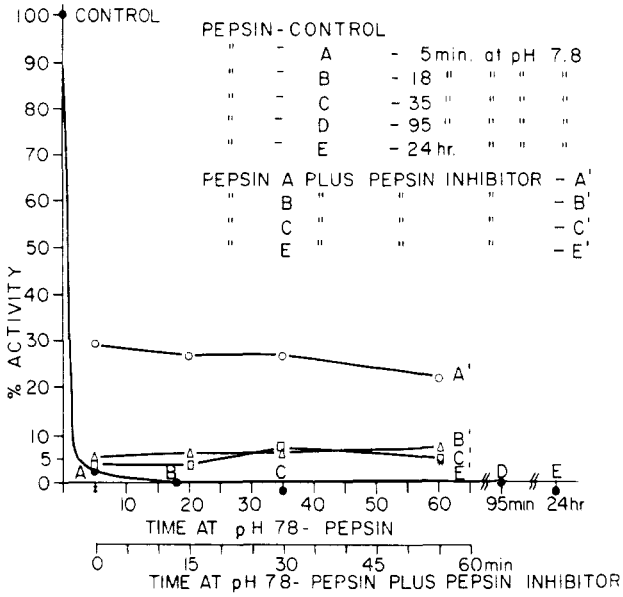

FIG. 10.—Loss of activity of pepsin at *p*H 7.8 and its partial restoration by pepsin inhibitor. See text for details.

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Reversible Dissociation of Aldolase into Unfolded Subunits*

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Dissociation of rabbit muscle aldolase into three highly unfolded subunits has been observed at *p*H values of 2.9 and below. These subunits appear to be of equal or nearly equal molecular weight. Reversal studies demonstrated that the subunits would, *in vitro*, reassociate and refold in a rapid, spontaneous, and specific manner upon neutralization. The sedimentation coefficient, catalytic activity, and immunological reactivity of the reassociated enzyme appear to be identical to those of native protein. This establishment, *in vitro*, of an enzyme structure of high molecular weight (150,000) and complexity (three chains) provides strong support for the hypothesis that the amino acid sequence of proteins uniquely determines their chain configuration and the spatial relationship of subunits. The mechanism of the process appears to involve, in both directions, an intermediate form. The available data are best explained by the assumption that this intermediate is a trimer of unfolded subunits.

The mechanism of the formation of globular proteins, which seemingly possess exceedingly complex and specific three-dimensional configurations, is a central problem in biochemistry. Much information has been gained concerning the way in which the amino acid sequence is dictated and formed, but much less attention has been paid to the equally important question of how the molecular configuration is prescribed. Especially interesting in this respect are the multichain enzymes, in which not only the folding of individual chains but the assembly of a definite number of these chains (in a specific spatial relationship) into the biologically active unit must be explained.

An important glycolytic enzyme, aldolase, has been shown to be of the multichain type. This has been demonstrated both by the detection of three carboxyl end-groups (Kowalsky and Boyer, 1959) and by dissociation of the enzyme by a detergent (Ramel *et al.*, 1961). Since the native protein has been reported (Taylor and Lowry, 1956) to have a molecular weight of 149,000, dissociation and reassociation experiments should provide a critical test of the hypothesis that in the biosynthesis of proteins the folding and association are dictated by the amino acid sequence. Also, the splitting of such a large unit into subunits offers considerable advantage in its further study.

Early observations by Gralen (1939) suggested that the dissociation of aldolase into its constituent peptide chains might be accomplished in acidic solutions. The use of such a mild and easily reversed environmental condition seemed a promising line of approach.

A preliminary report of our results has been given (Deal and Van Holde, 1962). Concurrently, similar studies have been carried out by Stellwagen and Schachman (1962a,b).

Experimental

Reagents and Buffer Solutions.—Fructose-1,6-diphosphate was obtained from K and K Laboratories, DPNH from Pabst Laboratories, and α-glycerophosphate dehydrogenase (containing triose phosphate isomerase) from C. F. Boehringer and Soehne. All other chemicals were of the best available commercial preparations.

All of the buffer solutions which were used were prepared from distilled deionized water through which nitrogen gas had been bubbled. Buffer solutions contained 0.05 M NaCl and 0.002 M EDTA, in addition to the appropriate amounts of sodium citrate and citric acid.

Aldolase.—Crystalline rabbit muscle aldolase was prepared by the method of Taylor (1955), with the modifications suggested by Kowalsky and Boyer (1959). Protein concentrations were determined spectrophotometrically with use of Taylor's (1955) value $E^{0.1\%}_{1\text{ cm}} = 0.91$ at 280 mμ. Assays of enzymatic activity were made by the hydrazine test (Jagannathan *et al.*, 1956; Richards and Rutter, 1961) and a coupled enzyme assay (Racker, 1947; Richards and Rutter, 1961). For immunologic tests, the Ouchterlony (Kabat and Mayer, 1961) technique of double diffusion on agar plates was used.

Sedimentation and Electrophoresis.—A Spinco Model E ultracentrifuge, equipped with a phase plate schlieren diaphragm and RTIC unit, was employed. All sedimentation experiments utilized Kel-F or filled-Epon centerpieces. Sedimentation velocity experiments were at rotor speed settings of 52,640 rpm, while sedimentation equilibrium experiments were generally carried out at 12,590 rpm (for the subunits) or 7,447 rpm (for the native aldolase). In the sedimentation equilibrium studies, short liquid columns (about 1.6 mm) were used

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