Two further conclusions may be stated more positively. First, the high molecular weights found for a very considerable portion of the solubilized protein, in conjunction with the functional group analyses previously reported,[1] show that extensive hydrolysis is not a requirement in the initial steps of the collagen–(acid) gelatin transition. Second, stable thermally solubilized proteins may be obtained which are similar to intact collagen and which might properly be called solubilized collagens. The term "parent gelatin," as applied to low molecular weight gelatins, lacks any real significance as applied in the degradation scheme.

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Intrinsic Viscosity and Optical Rotation of Proteins in Acid Media[1]

BY JEN TSI YANG AND JOSEPH F. FOSTER[2]

RECEIVED OCTOBER 7, 1954

Bovine plasma albumin is subject to gross, reversible structural alterations upon reduction of the *p*H of its solutions. Ovalbumin, on the other hand, does not show this structural versatility. A preliminary survey has now been made of the behavior of ten additional proteins under similar conditions. Of the proteins studied only one, namely, γ-globulin (human fract. II) shows definite evidence of a pronounced structural expansion in acid. Some possible implications of this fact in immunochemistry are discussed. The absence of evidence for structural variations at low *p*H in the case of the enzymes is discussed from the viewpoint of a current theory of enzyme activation based on the concept that enzymes must be capable of structural fluctuations. It is concluded that the results cannot be taken as evidence against this theory and the possibility that enzymes may show such effects under other conditions, particularly at alkaline *p*H, is raised.

There has been a recent upsurge in interest in the effect of *p*H on the optical activity and intrinsic viscosity of proteins. The specific rotations of a number of proteins as a function of *p*H and of denaturation have been reported in a sequence of papers by Jirgensons and co-workers.[3] Macheboeuf, *et al.*,[4] have demonstrated the pronounced *p*H dependence of the specific viscosity of horse serum albumin solutions. Golub and Pickett[5] have recently reported data on the *p*H dependence of the optical rotation of a number of common proteins.

The authors have recently shown that in the case of bovine plasma albumin there are marked increases in both specific rotation and intrinsic viscosity upon acidification.[6] The alterations appeared to be instantaneous, or nearly so, and fully reversible. The absence of any measurable streaming birefringence at low *p*H was taken as evidence that no pronounced unfolding of the protein molecule is involved but rather a molecular expansion. The closely parallel character of the shifts in the two properties was taken as evidence that the expansion is not gradual or stepwise, but rather an all-or-none transition from a condensed (native) to an expanded form. The viscosity increase, when extrapolated to zero protein concentration and zero ionic strength, is of such magnitude as to suggest an expansion in volume of the order twenty-fold.

Under the same conditions, it was shown that ovalbumin does not undergo a comparable alteration. Similar contrasts in the behavior of these two proteins have been demonstrated in urea denaturation by Kauzmann and co-workers.[7] Bovine plasma albumin and ovalbumin might thus be pictured as representing two extreme types of protein, the one being pliable and readily subject to reversible alterations in configuration, the other being tightly cross-linked so that any structural alterations take place only explosively and irreversibly.

It appeared of interest to survey the behavior of a number of proteins under the same experimental conditions employed in our earlier studies on plasma albumin and ovalbumin. It seemed particularly desirable to examine as many crystalline enzymes as possible in view of suggestions that pronounced reversible alterations in protein structure might be an inherent part of enzymic activation.[8]

Materials and Methods

Human γ-globulin, in the form of whole plasma fraction II, was supplied through the courtesy of Dr. J. N. Ashworth of the American Red Cross (sample designated as Squibb Lot No. 1201). Crystallized zinc insulin, pepsin, trypsin, chymotrypsin, chymotrypsinogen and ribonuclease were obtained from Armour and Company, and crystallized trypsinogen from Mann Research Laboratories, Inc. Three-times crystallized β-lactoglobulin was kindly donated by Drs. T. L. McMeekin and William G. Gordon of the Eastern Regional Research Laboratory. Three-times crystallized lysozyme was prepared earlier in this Laboratory by Dr. E. G. Samsa according to the procedure of Alderton and Fevold.[9] It has been stored for approximately two years at 2° and appeared to be approximately 98% pure by electrophoresis at *p*H 7.8.

All other chemicals employed were of reagent grade.

Protein samples were dissolved in distilled water, dilute HCl or dilute NaCl solution and dialyzed two or three times against large volumes of the same solvent. Prior to use,

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(3) B. Jirgensons, *Arch. Biochem. Biophys.*, **39**, 261 (1952); *ibid.*, **41**, 333 (1952); B. Jirgensons and S. Sirotzky, THIS JOURNAL, **76**, 1367 (1954).

(4) E. Gavrilesco, E. Barbu and M. Macheboeuf, *Bull. soc. chim. biol.*, **32**, 924 (1950); S. Bjornholm, E. Barbu and M. Macheboeuf, *ibid.*, **34**, 1083 (1952).

(5) M. Golub and E. Pickett, *J. Polymer Sci.*, **13**, 427 (1954).

(6) J. Yang and J. Foster, THIS JOURNAL, **76**, 1588 (1954).

(7) R. Simpson and W. Kauzmann, *ibid.*, **75**, 5139 (1953); J. Schellman, R. Simpson and W. Kauzmann, *ibid.*, **75**, 5152 (1953); W. Kauzmann and R. Simpson, *ibid.*, **75**, 5154 (1953); H. Frensdorff, M. Watson and W. Kauzmann, *ibid.*, **75**, 5157 (1953).

(8) K. Laidler and J. Hoare, *ibid.*, **72**, 2489 (1950).

(9) G. Alderton and H. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).

they were filtered through fine sintered-glass filters under pressure to remove traces of denatured and precipitated protein. Protein concentrations were calculated from micro-Kjeldahl nitrogen analyses using the following nitrogen composition values (taken from the literature): γ-globulin, 16.0%; zinc insulin, 15.5; pepsin, 14.6; trypsin, 15.0; trypsinogen, 15.0; chymotrypsin, 15.5; chymotrypsinogen, 15.8; ribonuclease, 16.0; β-lactoglobulin, 15.6; and lysozyme 18.6.

The experimental methods employed were the same as in the previous paper.[6] Since in that study it was shown that the expansion equilibrium is markedly suppressed by increasing ionic strength, all experiments except those with γ-globulin near the isoelectric point were conducted at the lowest possible ionic strength, *i.e.*, without addition of salt. In a very few experiments, undialyzed protein was employed, as noted in the captions to the figures. In such experiments, traces of extraneous ions were doubtless present.

Experimental Results

Serum γ-Globulin.—The results on γ-globulin are reported in Figs. 1, 2 and 3. Figure 1 shows plots of reduced viscosity *versus* concentration at various *p*H values, and Fig. 2 gives the corresponding data on optical rotation. In Fig. 3, the extrapolated values from Figs. 1 and 2 are given as a function of *p*H.

It is to be noted that marked changes in both the intrinsic viscosity and optical rotation of this protein occurred upon reduction of the *p*H of the solution. The changes are qualitatively very similar to those previously reported for bovine plasma albumin.[6] The rotational shift is seen to be of about the same magnitude, the viscosity increase somewhat smaller than in the plasma albumin case. A noteworthy difference is the fact that the two alterations do not parallel one another to the same degree in the case of γ-globulin.

That the alterations are reversible was demonstrated as in the previous case by removal of the acid through dialysis or by addition of salt. The optical rotation results are in reasonably good agreement with those of Jirgensons and Sirotzky.[3]

At very low *p*H, for example at *p*H 1.4, the protein was found to undergo an irreversible change accompanied by aggregation. Both viscosity and optical rotation were time dependent under such conditions, and finally the solutions became too turbid for measurements. This process may account for the failure of the optical rotation *vs.* *p*H curve to turn downward in the same *p*H region at which the viscosity decreases (Fig. 3).

Other Proteins.—The results obtained with zinc insulin, β-lactoglobulin and the enzymes and enzyme precursors are summarized in Figs. 4, 5, 6 and 7. It is to be noted that virtually no *p*H dependence is manifested in either property in any of these cases except for a possible viscosity effect with pepsin and probable rotational shifts with chymotrypsinogen and chymotrypsin.

Discussion

Plasma γ-Globulin.—The results would appear to demonstrate rather conclusively that γ-globulin is subject to pronounced reversible structural alterations as the *p*H of its solutions is altered. While the material utilized was whole fract. II of plasma, such material is typically 95% or more γ-globulin so that it seems most unlikely that the large variations observed can be due to protein species other than those of the γ-globulin type.

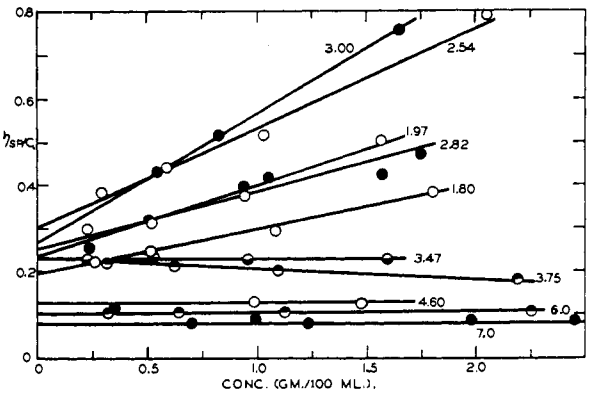

Fig. 1.—Reduced viscosity–concentration plots for human γ-globulin at various values of *p*H. The experiments at *p*H 6.0 and 7.0 utilized undialyzed protein. In the latter (*p*H 7) experiment 0.5 *M* NaCl was also added.

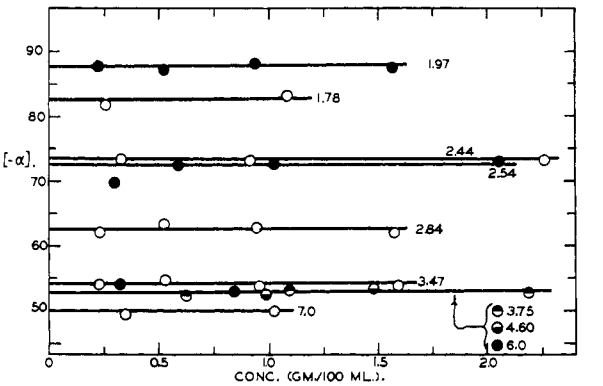

Fig. 2.—Specific rotation–concentration plots for human γ-globulin at various values of *p*H. The experiments at *p*H 6.0 and 7.0 were conducted on undialyzed protein. In the latter (*p*H 7) experiment 0.5 *M* NaCl was also added.

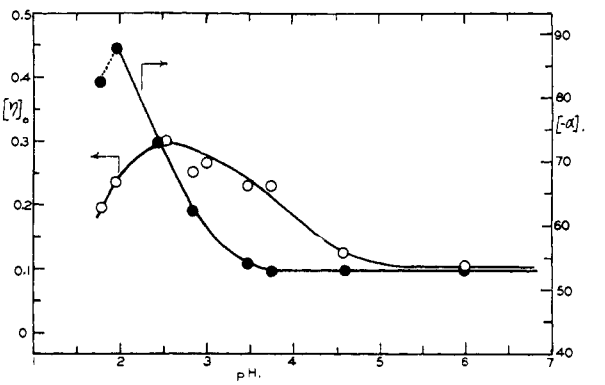

Fig. 3.—Dependence of intrinsic viscosity and limiting specific rotation of human γ-globulin on *p*H.

In view of the fact that the γ-globulin fraction of plasma contains most, if not all, of the plasma antibodies, these results raise many interesting possibilities and problems. Admittedly no reliable estimate can be given as to what portion of this fraction is active antibody. If it be accepted, for sake of argument, that most or all of the γ-globulin is active antibody, it follows that antibodies must be adaptable proteins of the plasma albumin type. This conclusion is a little difficult to accept in the

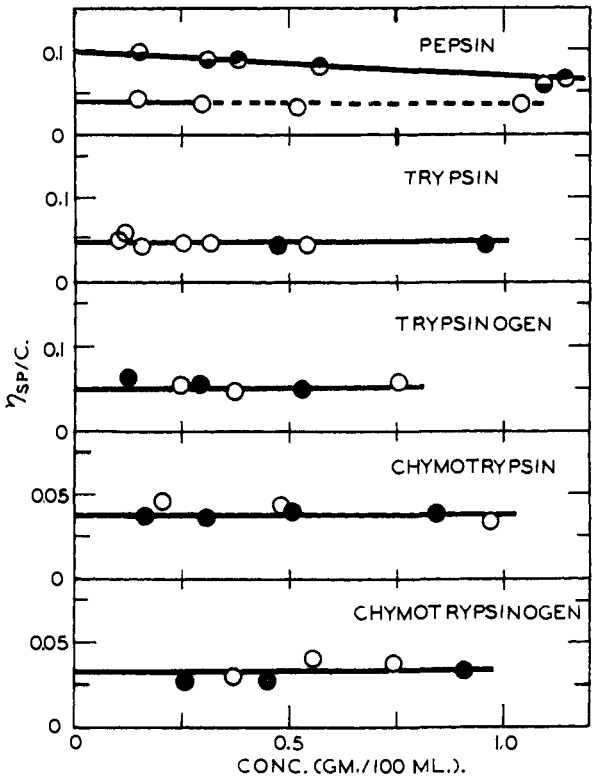

Fig. 4.—Reduced viscosity–concentration plots for several proteins at various *p*H values. Pepsin: ○, *p*H 2.3; ◒, *p*H 4.5; ◓, *p*H 4.6. Trypsin: ○, *p*H 3.1; ●, *p*H 3.0 (undialyzed). Trypsinogen: ●, *p*H 2.3; ○, *p*H 2.8. Chymotrypsin: ●, *p*H 2.5; ○, *p*H 5.7. Chymotrypsinogen: ●, *p*H 2.4; ○, *p*H 5.8.

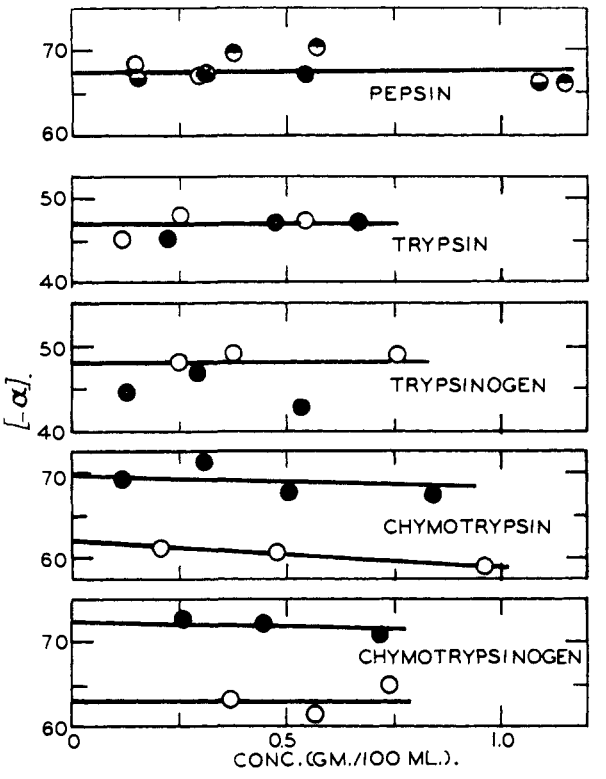

Fig. 5.—Specific rotation–concentration plots for several proteins at various *p*H values. Symbols used are the same as in Fig. 4.

face of rather well-accepted ideas as to the character of antibody–antigen specificity. Thus it would appear desirable that the specific configuration of antibodies be firmly fixed and not subject to chance local fluctuations in environment. The superficial observation that the rotational and viscosity alterations are readily reversible by no means can be taken as evidence that the initial detailed configuration is fully restored. On the other hand, perhaps configurational fluctuations are important in antibody–antigen combination and do not involve loss in specificity. It is to be hoped that some of these interesting possibilities basic to immuno-chemistry will be explored in the future.

Wright and Schomaker[10] have suggested a "reversibly protected" form of antibodies to account for their observed kinetics in urea inactivation. It seems possible that the acid-expanded form may be identical with their protected species. The authors have recently made this suggestion[11] in the case of bovine plasma albumin to account for the decreased rate of irreversible denaturation at low *p*H.

Insulin.—The low viscosities and rotations obtained at low *p*H indicate that insulin does not expand in acid solution. Golub and Pickett[6] obtained evidence for a slight but appreciable rotational increase with insulin at somewhat lower *p*H (around 2.0), and a much more pronounced increase in alkaline solution. Insulin is of interest in view of the additional complication of molecular dissociation which is well known in this case. It would appear that if dissociation is accompanied by a rotational increase, it is slight at most. There is evidently no molecular expansion accompanying dissociation. It might be postulated that in this case coulombic repulsion is relieved by dissociation rather than expansion. This suggests that globular proteins might be divided into three classifications on the basis of their response to extreme *p*H, namely, (1) those which retain their structural integrity, such as ovalbumin, (2) those which expand, such as plasma albumin and plasma γ-globulin, and (3) those which dissociate, such as insulin.

β-Lactoglobulin.—Both the viscosity and optical rotation remain low in acid solution in this case. From the standpoint of low *p*H behavior β-lactoglobulin should fall in the first classification (above). On the other hand, Golub and Pickett[5] found a pronounced increase in optical rotation for this protein on the alkaline side of the isoelectric point. Kauzmann, *et al.*,[7] also, have concluded this protein to lie between ovalbumin and plasma albumin with respect to their behavior toward urea.

Enzymes.—The behavior of enzymes with respect to structural variability is of special interest for reasons mentioned in the introduction. Thus, if enzymes have the facility of undergoing structural alterations in conjunction with the substrate activation process it would seem reasonable that they should do so under the influence of strong coulomb repulsions, *i.e.*, they should belong to group (1)

(10) G. Wright and V. Schomaker, THIS JOURNAL, **70**, 356 (1948).
(11) J. F. Foster and J. T. Yang, *ibid.*, **77**, in press (1955).

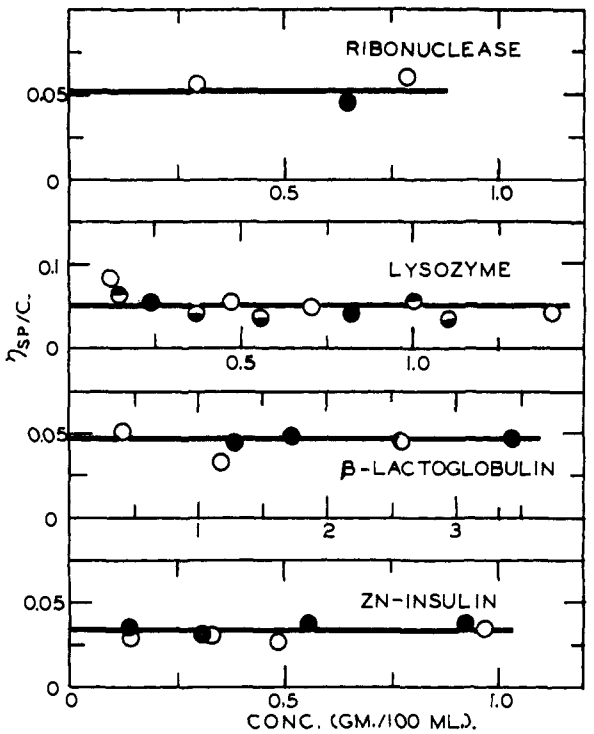

Fig. 6.—Reduced viscosity–concentration plots for several proteins at various *p*H values. Ribonuclease: ○, *p*H 2.5; ●, *p*H 3.8. Lysozyme: ◒, *p*H 2.3; ◓, *p*H 3.1; ○, *p*H 7.0; ●, *p*H 7.5. β-Lactoglobulin: ○, *p*H 1.7; ●, *p*H 2.6. Zinc insulin: ●, *p*H 2.3; ○, *p*H 2.8 (undialyzed).

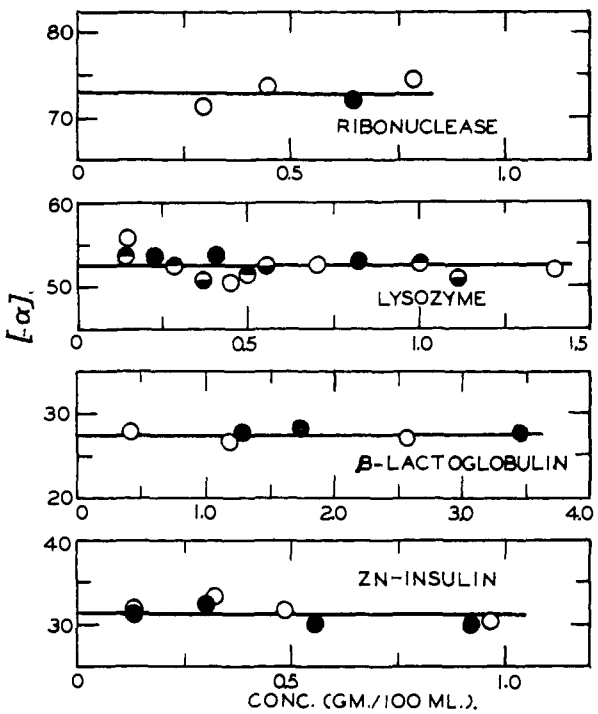

Fig. 7.—Specific rotation–concentration plots for several proteins at various *p*H values. Symbols used are the same as in Fig. 6.

above. From this standpoint, the results reported here must be considered, on the whole, as negative. None of the enzymes, nor the enzyme precursors, show any clear evidence for structural alteration in acid solution. The increased viscosity of pepsin at *p*H 4.5 as compared to 2.3 may or may not be significant. It is not backed up by any variation in optical rotation. It should be pointed out that in this case, since the isoelectric point of the protein is very low, the minimum rotation and viscosity might be expected at very low *p*H. From this point of view, the increased viscosity at *p*H 4.5 is in the expected direction. Golub and Pickett[5] also found no *p*H variation in the optical rotation of pepsin in acid solution, but found a marked increase above *p*H 7. Unfortunately, their specific rotation in acid solution appears to be significantly higher than the value found in the present study.

In the case of trypsin, our optical rotation values at low *p*H are in serious disagreement with those of Golub and Pickett.[5] Our value near *p*H 3 is close to the value they found at *p*H 5–7, whereas they found a substantial increase below *p*H 4. No explanation can be offered for this lack of agreement. Again the low intrinsic viscosity at low *p*H indicates that at least no pronounced molecular expansion has taken place. It should be pointed out that in this case, too, Golub and Pickett found an increase in rotation in alkaline solution.

The increased rotation of chymotrypsin and chymotrypsinogen in acid solution appears to be clearly significant, although it is not accompanied by any viscosity increase. The close similarity in behavior of enzyme and enzyme precursor, with respect to both of the properties, is noteworthy.

The absence of evidence for marked structural alteration in enzymes in these studies certainly should not be taken as in any sense evidence against the concept of structural fluctuation in enzymic activation. In the first place, these studies are limited to the acid side of neutrality and molecular expansion may well take place in some of the cases in alkaline solution. As has been pointed out above, in the case of several of the enzymes the studies of optical rotation by Golub and Pickett[5] indicate the possibility of such changes. Furthermore, as has been pointed out by Lumry and Eyring,[12] the volume and entropy changes associated with the activation process are not of large enough magnitude to require drastic configurational changes in the enzyme structure. It thus may be naive to expect enzymes as a class to show the gross structural alterations exhibited by plasma albumin even if this conception of enzyme activation be the correct one.

Finally, reference should be made to a suggestion of Kauzmann, *et al.*,[13] concerning the possible existence of a correlation between the specific rotation of proteins at their isoelectric points and their structural rigidity. The general idea expressed is that those proteins with abnormally high isoelectric rotations, such as plasma albumin, are characterized by large numbers of cross linkages which prevent their packing in a tight, rigid structure, and they are hence structurally unstable or adaptable. The finding that γ-globulin is subject to

(12) R. Lumry and H. Eyring, *J. Phys. Chem.*, **58**, 110 (1954).

(13) W. Kauzmann in McElroy and Glass, "The Mechanism of Enzyme Action," The John Hopkins Press, Baltimore, Md., 1954, p. 70.

pronounced structural alterations in acid media is in accord with this idea since it also possesses a high isoelectric rotation (above $-50°$). The structural rigidity of β-lactoglobulin and insulin, in this study, is also in accord since they have very low rotations in the isoelectric state. However, by the same token, it would be predicted that most of the enzymes should be structurally adaptable, particularly pepsin and ribonuclease which have specific rotations of around $-70°$. Further studies of these proteins in alkaline solution, and perhaps also in urea, would clearly be in order.

Acknowledgment.—We are indebted to Miss Ana Pellecer for her technical assistance in carrying out a large proportion of the experimental measurements. We would also like to express our thanks to Drs. J. N. Ashworth, T. L. McMeekin, W. G. Gordon, J. B. Lesh and E. G. Samsa for supplying protein samples.

AMES, IOWA

[CONTRIBUTION NO. 1938 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Re-evaluation of the Inhibition Constants of Previously Investigated Competitive Inhibitors of α-Chymotrypsin. I. Hydrolysis Products and Enantiomorphs of Previously Investigated Specific Substrates[1]

BY ROBERT J. FOSTER, HENRY J. SHINE AND CARL NIEMANN[2]

RECEIVED SEPTEMBER 9, 1954

The enzyme–inhibitor dissociation constants of α-chymotrypsin and five hydrolysis products and ten enantiomorphs of previously investigated specific substrates of this enzyme have been re-evaluated from primary data by procedures which are more objective than those used previously and more reliable and realistic values for these constants have been obtained.

It is to be expected[3,4] that an α-chymotrypsin-catalyzed hydrolysis of a given specific substrate when conducted under zone A conditions[4-6] may be competitively inhibited by one or more of the hydrolysis products and that such a reaction can be described throughout its course, in so far as it is dependent upon the concentration of enzyme and specific substrate, in terms of equations 1, 2, 3 and 4 where $K_S = (k_2 + k_3)/k_1$, $K_{P_1} = k_5/k_4$ and $K_{P_2} = k_7/k_6$.

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \quad (1)$$

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \quad (2)$$

$$E_f + P_{2f} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EP_2 \quad (3)$$

$$k_3[E]t = K_S\left(1 + [S]_0 \sum_{j=1}^{n} 1/K_{P_j}\right) \ln [S]_0/[S]_t + \left(1 - K_S \sum_{j=1}^{n} 1/K_{P_j}\right)([S]_0 - [S]_t) \quad (4)$$

In previous studies conducted in these laboratories[3,7-11] it was shown that with a number of acylated α-amino acid amides and hydroxamides only one of the hydrolysis products, *i.e.*, the carboxylate ion derived from the acylated α-amino acid, may competitively inhibit the hydrolytic reaction and that the other hydrolysis product, *i.e.*, ammonia or hydroxylamine or the corresponding monoprotonated species, is without effect even when present in concentrations which are considerably greater than those of the specific substrate. Thus, with knowledge of the K_{P_1} value of the carboxylate ion of the acylated α-amino acid, and the K_S and k_3 values of the corresponding specific substrate, it is possible to describe, within the limits of experimental error, the α-chymotrypsin-catalyzed hydrolysis of a number of specific substrates over a substantial portion of the reaction in terms of equation 4.[3,7,8,10,11] To date we have reported[3,7-10] K_{P_1} values for the carboxylate ions derived from six acylated α-amino acids which are hydrolysis products of eight of the sixteen specific substrates of α-chymotrypsin which have been studied in these laboratories and for which revised values of K_S and k_3 are now available.[4] Since the above K_{P_1} values were determined by procedures which can now be questioned[4] we have, in this study, re-evaluated these constants from the original primary data by more objective procedures than those used previously and thus have obtained a set of more reliable and realistic constants.

Most of the primary data available for the re-evaluation of the enzyme–inhibitor dissociation constants of α-chymotrypsin and the carboxylate ions of the various acylated α-amino acids relate to the situation obtaining during the initial stages of hydrolysis of a particular specific substrate in the presence of known amounts of added inhibitor. Therefore, these primary data were re-evaluated through the use of the procedure proposed by Jennings and Niemann[12] and the initial velocities so

(1) Supported in part by a grant from Eli Lilly and Co.

(2) To whom inquiries regarding this article should be sent.

(3) R. J. Foster and C. Niemann, *Proc. Nat. Acad. Sci.*, **39**, 999 (1953).

(4) R. J. Foster and C. Niemann, THIS JOURNAL, **77**, 1886 (1955).

(5) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).

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(7) H. T. Huang and C. Niemann, THIS JOURNAL **73**, 1541 (1951).

(8) D. W. Thomas, R. V. MacAllister and C. Niemann, *ibid.*, **73**, 1548 (1951).

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(11) D. S. Hogness and C. Niemann, *ibid.*, **75**, 884 (1953).

(12) R. R. Jennings and C. Niemann, *ibid.*, **75**, 4687 (1953).