with a resulting elimination of water from a number of the charged groups as the surfaces come together.

This explanation is confronted by one obvious difficulty. The entropy of activation is much larger and more positive for dimerization with the bifunctional organic mercurial than for the formation of the simple mercury dimer. On the other hand, the latter requires a much closer approach of the two albumin molecules and, therefore, might be expected to involve a larger surface region of close contact between the two mercaptalbumin molecules than the former. Thus, the magnitude of the dehydration effects should, if anything, be greater for the formation of ASHgSA than for that of ASHgRHgSA, and the entropy of activation in dimer formation should be greater for the former. The experimental facts indicate just the contrary. However, so little is known of the details of the process that this argument cannot be taken as a definite refutation of the explanation of the positive value of $\Delta S^{\ddagger}$ in terms of dehydration.

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[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

A Disulfide Dimer of Human Mercaptalbumin[1a,b]

BY RUDOLF STRAESSLE[1c]

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Human serum mercaptalbumin (Alb-SH) forms a disulfide dimer (Alb-S-S-Alb) which is prepared by oxidation with iodine of the mercury dimer (Alb-S-Hg-S-Alb). Maximum yield of the disulfide dimer is obtained when the reaction is carried out at low temperature (−8 to −18°), the protein being in the solid state in a system containing a high concentration of ethanol. The maximum yield of disulfide dimer obtained was 75–80%. Measurements of sedimentation constant and light scattering indicated that the disulfide dimer had approximately the same size and shape as the mercury dimer. Fractionation procedures gave some separation of dimer from monomer, but a completely pure disulfide dimer preparation has not yet been achieved. Unlike the mercury dimer, the disulfide dimer is not dissociated by the addition of an excess of a mercuric salt or of iodide ion. Reduction with cysteine, however, reconverts Alb-S-S-Alb to two moles of Alb-SH as shown by the change of sedimentation constant to that of the monomer and by the reappearance of titratable sulfhydryl groups.

Introduction

Human serum mercaptalbumin contains one and only one sulfhydryl group per molecule[2,3] and forms a mercury dimer of the composition $(Alb\text{-}S)_2\text{-}Hg$– the symbol Alb-SH being used to denote mercaptalbumin.[2–4] In the dimer, the two albumin molecules are linked through an S–Hg–S bridge, involving a cysteinyl residue in each molecule.

In the course of studies on the iodination of human serum albumin[5] it was found that when using the mercury dimer of mercaptalbumin instead of the monomer form of human serum albumin as starting material, the formation of two components was observed. Ultracentrifugal analysis indicated that one corresponded to monomer albumin and the other to a component with a sedimentation constant virtually identical with that of the mercury dimer. The double molecule, however, did not dissociate upon addition of one mole of mercuric chloride, although this reagent immediately dissociates the mercury dimer.[4] This suggested that iodine had oxidized the two adjoining sulfhydryl groups of the mercaptalbumin molecule to a disulfide bond with the release of mercuric iodide. While the actual process may proceed in several steps, the over-all reaction should presumably be formulated as

$$\text{Alb-S-Hg-S-Alb} + I_2 = \text{Alb-S-S-Alb} + HgI_2 \quad (1)$$

Oxidation of mercaptides of low molecular weight by halogens generally leads to the formation of disulfides.[6] Under rather special conditions, the formation of the corresponding sulfenyl iodide has been observed.[7] Sulfenyl halides, however, tend to form disulfides in many ways; among others by hydrolysis, alcoholysis and reaction with mercaptans. The over-all reaction, therefore, tends to be the formation of disulfide. The disulfide linkage, once formed, has been reported by Kharasch[6] to be cleaved only by a considerable excess of halogen. Formation of a disulfide–tetraiodide may occur as a side-reaction.

It seemed reasonable, therefore, to assume, as was further suggested by the experimental results, that a disulfide dimer was formed in a side-reaction during the iodination of the mercury albumin dimer. This reaction was the subject of the investigation here presented.

Materials and Methods

Crystallized Mercury Albumin Dimer.—One sample (prep. 178–5x) was crystallized according to Hughes[2] as the mercuric salt from human serum albumin, and subsequently recrystallized three times from ethanol–water mixtures of the composition: EtOH, 7.5%; *p*H 5.1; Γ/2 0.01; T −5°; protein concn., 13%. The preparation was analyzed in the ultracentrifuge as a 0.7% solution at *p*H 6.50 and Γ/2 0.15. It consisted of 95% dimer ($s_{20,w}$ = 6.5*S*) and 5% monomer sedimenting with the rate characteristic

(1) (a) This work has been supported by funds of Harvard University and the Eugene Higgins Trust, by grants from the Rockefeller Foundation and the National Institutes of Health, and by contributions from industry. (b) This paper is No. 106 in the series "Studies on the Plasma Proteins" from blood collected by the American Red Cross, on products developed by the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University. (c) F. Hoffmann–La Roche and Co. A.G., Basle, Switzerland, Research Laboratories.

(2) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947).

(3) W. L. Hughes, Jr., Cold Spring Harbor Symp. on Quant. Biol., **14**, 79 (1949).

(4) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).

(5) W. L. Hughes, Jr., and R. Straessle, *ibid.*, **72**, 452 (1950).

(6) N. Kharasch, S. J. Potempa and H. L. Wehrmeister, *Chem. Revs.*, **39**, 269 (1946).

(7) H. Rheinboldt, *et al.*, *Rev. brasil chim.*, (*São Paulo*), **4**, 169 (1937); *C. A.*, **32**, 484 (1938); *Ber.*, **72**, 657, 668 (1939).

of serum albumin. Another preparation of human serum albumin, Lot 352RR, was used as a starting material, and the mercury dimer obtained from it was recrystallized three times and designated as preparation 352-Hg-1. Its homogeneity was within the same limits.

Human Mercaptalbumin.—This was prepared from three-times recrystallized mercury albumin dimer (352-Hg-1) by removing the mercury through dialysis of 100 ml. of 20% protein solution against three changes of buffer, 500 ml. of each, consisting of an acetate buffer of *p*H 5.8 and Γ/2 0.05, 10^{-3} *M* in cysteine. The cysteine was then displaced by repeated dialysis against acetate buffer (*p*H 4.95, Γ/2 0.05), and finally against distilled water.[8] The preparation consisted of 95% of material sedimenting with the rate of human serum albumin ($s_{20,w}$ = 4.6*S*) besides some 3–5% of both faster and slower sedimenting material. It contained 0.95 sulfhydryl group per mole, titratable with methyl mercuric iodide.

For some experiments an older preparation was used which contained only 0.7 sulfhydryl group per mole (prepn. 3A). It contained 93% of material sedimenting at the characteristic rate for serum albumin with 7% of a faster sedimenting component. The preparation had been stored as a dry powder at 4° for a period of two years.

Estimation of the Yield of Disulfide Dimer.—The yield was calculated from ultracentrifugal analyses[9] carried out in an air-driven ultracentrifuge, equipped with a modified Philpot schlieren optical system, at a speed of 54,000 r.p.m. and at an average temperature of 24°. The sedimentation constants agreed well with the corresponding values found for the mercury albumin monomer and dimer.

For some experiments the light scattering method proved useful in estimating the changes in molecular size. The apparatus has been described by Edsall, Edelhoch, Lontie and Morrison[10]; see also Edelhoch, *et al.*[4] The measurements were carried out with 1% protein solutions, usually at *p*H 4.95 and Γ/2 0.05, in quartz cells of 1 × 1 cm. square cross section, and at 436 mμ.

Free Sulfhydryl Groups.—These groups were determined according to Hughes[3,4] by either one of two methods.

In the direct method one volume of 5–10% protein solution was denatured by the addition of two volumes of a 5.4 *M* guanidinium bromide solution, containing 0.5 mole of sodium carbonate, and then titrated with a 10^{-3} *M* solution of methyl mercuric nitrate in water, using a trace of sodium nitroprusside as indicator.

In the indirect method an aliquot of a 5% protein solution at *p*H 10 and 4° was equilibrated with an excess of 5×10^{-4} *M* methyl mercuric iodide in toluene, and the excess titrated back with a dithizone solution of the following composition: 3 mg. of dithizone dissolved in carbon tetrachloride, containing 3 ml. of abs. methanol, 0.2 ml. of amylamine and 0.3 ml. of glacial acetic acid in a total volume of 100 ml.

Experimental

Preparation of the Disulfide Dimer from the Mercury Dimer of Human Serum Albumin.—Three grams of mercury albumin dimer (352-Hg-1), corresponding to 0.217×10^{-4} mole of dimer,[11] were rapidly dissolved at 2° with the aid of 10 ml. of acetate buffer of *p*H 4.95 and Γ/2 0.12. The solution was added dropwise with stirring to 100 ml. of 95% ethanol, precooled to −18°. To the fine suspension was added 1.38 ml. of 0.0475 *N* iodine dissolved in 95% ethanol, an amount corresponding to 3 equiv. of iodine per mole of mercury dimer. The suspension was kept at −18° for 19 hours with stirring and then centrifuged in a refrigerated angle-centrifuge. The supernatant alcohol solution was discarded, the paste resuspended at −18° in 70 ml. of an acetate buffer (*p*H 4.95, Γ/2 0.02, ethanol 40%) and centrifuged again. The paste was then dissolved with the aid of 2 ml. of 2.5 *M* sodium acetate at −8°, the solution transferred to a cellophane bag at low temperature, dialyzed against an acetate buffer (*p*H 4.95, Γ/2 0.05 sodium acetate and 0.10 sodium chloride) and analyzed in the ultracentrifuge at a protein concentration of 1% with the result: monomer, 37%; dimer, 58%; faster sedimenting, 5%. This composition was found to remain unchanged even in the presence of 10 moles of potassium iodide per mole protein, which excludes the presence of mercury dimer in the mixture (see Edelhoch, Katchalski, *et al.*, ref. 4).

Variation of the Reaction Conditions. 1. Amount of Oxidant.—With the method just described, but using variable amounts of iodine, the yield of disulfide dimer as computed from ultracentrifugal analyses is given in Table I.

TABLE I

YIELD OF DISULFIDE DIMER AS A FUNCTION OF IODINE ADDED

Iodine added, equiv./Hg(SAlb)$_2$	Dimer, %
1	25
3	76
4	72
6	80

2. Type of Oxidant.—When aqueous solutions of hydrogen peroxide (*p*H 4.95, 7.05 and 10), arsenic acid (*p*H 5.8) or potassium permanganate were used as oxidants, little dimer formation was observed. Thiocyanogen, $(SCN)_2$,[13] unfortunately is very sensitive to traces of moisture. The commonly used solvent, carbon tetrachloride + 10% acetic anhydride/glacial acetic acid, would appear to exclude thiocyanogen as a useful reagent. The use of bromine (4 equiv./mole) instead of iodine resulted in a yield of only 29% disulfide dimer.

3. Effect of *p*H.—Since essentially the same yields of dimer were obtained at *p*H values of 4.95, 6.50 and 8.50, using 4 equiv. of iodine per mole of protein, the isoelectric protein was used in the subsequent experiments.

4. Duration of Oxidation.—In order to investigate whether or not the time of contact with the oxidant would have any influence upon the yield of disulfide dimer, a series of experiments was carried out under standard conditions varying only the reaction time. The results are reported in Table II.

TABLE II

YIELD OF DISULFIDE DIMER AS AFFECTED BY TIME OF CONTACT WITH OXIDANT

Duration	Iodine added, equiv./Hg(SAlb)$_2$	Dimer, %
20 min.	4	72
17 hr.	3	76
16 hr.	6	80

5. Ethanol Concentration.—Early experiments were performed in buffered solution at 2°, but the yield of dimer was found to be low. The experiments reported in Table III were carried out by suspending the protein in ethanol–water mixtures of varying composition. The temperature was −18° except for expts. 7 and 8, in which a temperature of −8° was chosen. The added iodine corresponded to 2 equiv./mole albumin in the first three experiments, and to 4 equiv./mole in the others. The time of contact with the iodine was not extended beyond one hour, except for nos. 7 and 8, where the iodine was allowed to react for 24 hours. In all experiments, at least 90% of the iodine was consumed after this period of time.

6. Temperature.—The temperature was held as low as possible in order to avoid serious side-reactions due to the high alcohol concentration. No difference in the yield could be observed if the reaction was carried out at either −8° or −18°. Table IV shows that the yield is decreased by lowering the temperature further to −86°, keeping the other reaction conditions as usual. At −86° the rate of

(8) More recently it has been found preferable to remove the mercury from the mercury dimer by the method of H. M. Dintzis (see ref. 4, and the refs. there given) adding thioglycolate which forms a mercury complex, and then removing this complex from solution by adsorption on Amberlite resin (IRA-400, 20–50 mesh).

(9) Ultracentrifugal analyses were carried out by Mr. Charles Gordon and computed by Mr. Frank Gordon and Mrs. Virginia Mannick under the supervision of Dr. J. L. Oncley.

(10) J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, THIS JOURNAL, **72**, 4641 (1950).

(11) We have taken the molecular weight of the albumin monomer, as 69,000 in this paper, although there are indications (see for instance Low[12]) that this figure may require some downward revision.

(12) B. W. Low, THIS JOURNAL, **74**, 4830 (1952).

(13) H. S. Booth, "Inorganic Syntheses," Vol. I, McGraw-Hill Book Co., Inc., New York, N. Y., 1939, p. 84.

TABLE III

YIELD OF DISULFIDE DIMER AS INFLUENCED BY THE WATER CONTENT OF THE SYSTEM

No.	Organic solvent	Water volume, %	Dimer, %
1	.....	100	11
2	Ethanol	65	27
3	Ethanol	50	40
4	Ethanol	50	32
5	Ethanol	18	72
6	Ethanol	6	78
7	Acetone	0	8[a]
8	CCl_4	0	0

[a] This figure may probably be taken as zero within the limits of experimental error.

disappearance of the iodine color was very slow. This may account for the low yield.

TABLE IV

YIELD OF DISULFIDE DIMER AS A FUNCTION OF TEMPERATURE

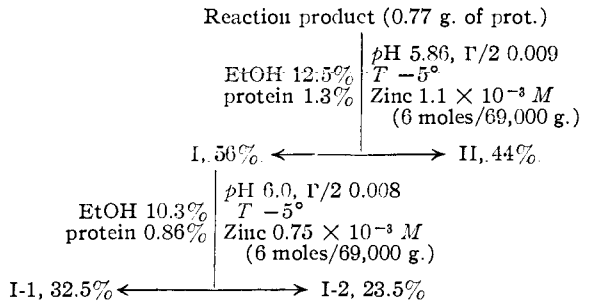
Temp., °C.	Duration	Dimer, %
−20	20 min.	72
−86	22 hr.	37

Fractionation of the Reaction Mixture.—The analytical data show that even under conditions where the best yield of disulfide dimer is obtained, the reaction mixture still contains 20–50% of the monomer component, presumably mercaptalbumin containing the sulfhydryl group in an oxidized form. In order to remove this component, a fractionation in the presence of zinc ions was carried out.

1. **Fractionation with 6 Moles of Zn/69,000 g. of Protein.**—The mixture containing the disulfide dimer was prepared from 2 g. of mercury albumin dimer (352-Hg-1) with 4 equiv. of iodine/mole, as described in the beginning of the Experimental section. The reaction product contained 50% of disulfide dimer, 46% of monomer, and a small amount of both slower and faster sedimenting material. It was finally dialyzed against an acetate buffer of *p*H 5.86 and Γ/2 0.01 with frequent changes. The solution containing 0.77 g. of protein was diluted to 50 ml. with the same buffer, 0.67 ml. of 10^{-2} *M* zinc acetate solution was added (6 moles of zinc/69,000 g. of protein), followed by 10 ml. of 75% ethanol at −5°. The precipitate was centrifuged off, the paste dissolved in a suitable volume of buffer and fractionated again. The procedure is represented in the scheme given in Chart I. Fractions I and I-1 were analyzed in the

CHART I

FRACTIONATION OF THE REACTION PRODUCT CONTAINING THE DISULFIDE DIMER



ultracentrifuge, dissolved in an acetate buffer (*p*H 4.95, Γ/2 0.05 acetate + 0.10 chloride). Fraction I-1 was then treated with 10^{-3} *M* cysteine for 22 hours at 4° (I-1, cyst.), and analyzed again. The results are summarized in Table V. The experiment shows that under the conditions applied a fraction can be obtained, containing about one-third the total protein, in which the relative concentration of the disulfide dimer is increased by 50% with respect to the amount initially present. Variation of zinc concentration from 10^{-3} *M* to 4×10^{-3} *M* had little effect upon the concentration of disulfide dimer in the fractions.

TABLE V

INCREASE IN RELATIVE CONCENTRATION OF DISULFIDE DIMER UPON FRACTIONATION

Fraction	Compositions of fractions Monomer, %	Dimer, %	Faster, %
Init.	46	50	4
I	28.5	61	10.5
I-1	15	73.5	11.5
I-1, cyst.	27	62	11

Reconversion of the Disulfide Dimer to Mercaptalbumin.—Reduction to mercaptalbumin with cysteine has yielded important evidence for the assumed formula of the disulfide dimer.

1. **Preparation of the Dimer.**—The dimer was prepared from 3 g. of mercury albumin dimer (352-Hg-1) with 3 equiv. of iodine/mole, as described before. The ultracentrifugal analysis showed the reaction product to consist of 37% monomer, 58% dimer and 5% faster sedimenting material. The sulfhydryl content was negligible.

2. **Reconversion with Cysteine.**—To 21 ml. of a 6.20% solution of this disulfide dimer preparation (1.9×10^{-5} mole), cysteine hydrochloride (3.8×10^{-4} mole) was added with an equimolar amount of sodium bicarbonate, and 1 ml. of a phosphate buffer of *p*H 7.05 and Γ/2 3.5. The final *p*H was 6.24, and the cysteine concentration 1.8×10^{-2} *M*. The solution remained at 2°, aliquots of 3 ml. being withdrawn at intervals and added dropwise at −18° to 30 ml. of buffered 49% ethanol (acetate buffer of *p*H 4.75 and Γ/2 0.03) in order to effect precipitation of the total protein. The samples were then centrifuged, dissolved in sodium acetate (0.001 *M*) and dialyzed for several days against distilled water, which was changed frequently. Table VI gives the sulfhydryl analyses.

TABLE VI

RECONVERSION OF THE DISULFIDE DIMER TO MERCAPTALBUMIN BY CYSTEINE

Sample no.	1	2	3	4	5	6	7	8	9
Cysteine-treated hr.	0	0.5	1	2	4	11	24	72	MA
SH/69,000 g.	0	0.38	0.53	0.62	0.72	0.81	0.89	0.90	0.91

Sample no. 9 represents mercaptalbumin, prepared from the mercury albumin dimer (352-Hg-1), in order to determine the maximum sulfhydryl content obtainable with this preparation. The sample which had been treated with cysteine for three days was analyzed in the ultracentrifuge in the usual acetate–chloride buffer of *p*H 4.95. It contained 96.6% of material with a sedimentation constant of 4.6*S*, the value corresponding to human serum albumin and only 3.4% of a faster sedimenting component.

Discussion

The formation of a disulfide dimer of human serum albumin has been shown to occur upon oxidation with iodine of the mercury dimer of this protein. Iodine reacts in various ways with proteins. In order to make the reaction specific for the oxidation of the sulfhydryl group in albumin, advantage has been taken of the observation that the sulfhydryl groups are the first groups in proteins to consume iodine.[4] According to the equation

$$\text{Alb-S-Hg-S-Alb} \xrightarrow{-2e} \text{Alb-S-S-Alb} + Hg^{++}$$

2 equiv. of iodine/mole of mercury dimer are required for oxidation to the disulfide. In accord with this mechanism, it has been found that at least 2 equiv. was required to obtain a satisfactory yield (Table I). Any great excess over this amount is likely to give rise to side-reactions; the sulfhydryl group may be oxidized further, other protein groups may be oxidized, or substitution may occur.

Since the yield was unchanged when the added iodine varied from 2–6 equiv./mole dimer, 3–4 equiv./mole have usually been used.

The type of oxidant is of particular importance. Several oxidants, such as H_2O_2, $KMnO_4$, $K_2Cr_2O_7$, have been found ineffective, perhaps because their oxidation–reduction potential is too high for the reaction under consideration, and they would probably oxidize the sulfhydryl group beyond the disulfide stage. Among the halogens, chlorine, and even bromine, may be excluded for the same reason. Thiocyanogen and arsenic acid also were found unsuitable.

The state of the protein appears to be of primary importance if adequate yields are to be obtained. Steric considerations require that, during oxidation, the sulfhydryl groups of two protein molecules remain properly oriented near one another for a long enough time to permit coupling. This condition does not occur with solutions of mercaptalbumin itself where iodine oxidation always goes beyond the disulfide stage to an oxidation state corresponding to RSOH.[5] Orientation of pairs of sulfhydryl groups has, therefore, been achieved by coupling them in the mercury dimer of mercaptalbumin. Even so, yields were poor unless the orientation was further stabilized by carrying out the reaction with the protein in the solid state (Table III). Maintenance of the mercury dimer in the solid state is not a sufficient condition as evidenced by experiments 7 and 8 (in Table III) where acetone and carbon tetrachloride were used to keep the protein insoluble.

The highest yields, obtained in a solvent of 72–94% ethanol by volume, may be partly due to a side-reaction. It has been found that treatment of mercaptalbumin with these concentrations of ethanol alone causes the formation of a new component with a sedimentation constant typical of albumin dimer.[14] This component is formed in amounts up to 30% of the total albumin at alcohol concentrations above 60% by volume. When the sulfhydryl group of mercaptalbumin was blocked by iodoacetamide, however, and the protein now subjected to the alcohol treatment, no faster sedimenting component appeared, as indicated both by ultracentrifugal and light scattering analysis. This seems to exclude mere denaturation in the formation of this component and rather suggests that the amount of oxygen dissolved in the mixture (which is greater at the low temperature than at room temperature) is sufficient to oxidize some 30% of mercaptalbumin to the disulfide dimer.[16]

The influence of *p*H on the yield of stable dimer was found to be negligible over the *p*H range 5–8.5. The rate of reaction at −18° appears to be quite high, since the yield of disulfide dimer was as high as 50–70% at the end of 15 minutes. At −86° the rate of reaction became too slow to be practical. Intermediate temperatures have not been investigated.

The absence of sulfhydryl groups in the reaction mixture, the presence of two distinct components, as revealed by ultracentrifugal analysis, and the agreement of their sedimentation behavior with that of mercaptalbumin and its mercury dimer—all these facts provide evidence that the reaction leads to the expected disulfide dimer. That some side-reactions do occur, might be inferred from the appearance of faster sedimenting material.

Additional important evidence for the disulfide character of the dimer is provided by the reversal of the reaction in the presence of cysteine; the reaction yielding one free sulfhydryl group per mole of albumin, with disappearance of the faster sedimenting component, and a corresponding increase in the amount of the monomer. The concentration of cysteine was found to be important. In 10^{-3} *M* cysteine, under otherwise similar conditions, only 16% of the amount of disulfide dimer initially present was converted to monomer within 24 hours. In 10^{-2} *M* cysteine, the reaction apparently went to completion.

The disulfide dimer thus behaved quite differently from the mercury dimer, which is completely dissociated within a few seconds by one mole of cysteine per mole of dimer, corresponding to a cysteine concentration of about 10^{-4} *M* in a 1.4% protein solution. The disulfide dimer required at least 24 hours, and a 100-fold increase in the concentration of cysteine for its reversion.

During the course of these reversion experiments, the influence of higher cysteine concentrations upon albumin was also studied. If the treatment was carried out at a cysteine concentration of 0.1 *M* at 2°, *p*H 6.9 for 12 days, followed by subsequent prolonged dialysis against buffer in order to displace the cysteine, the sulfhydryl content became as great as 1.6 groups/mole. The value may have been even higher in the presence of the cysteine, since some reoxidation is likely to have taken place during dialysis. This seems to indicate that, besides the free sulfhydryl group present in normal mercaptalbumin, at least one of the disulfide linkages, present in native albumin, must have been cleaved in a fraction of the protein. Since there are originally 16 disulfide linkages[17] in human serum albumin, their great stability toward cysteine at this *p*H is apparent, because no value greater than 1.6 groups/mole was ever observed in the course of these experiments.[18]

The most favorable conditions yet found for the preparation of the disulfide dimer are: One volume of a 30% mercaptalbumin mercury dimer solution at *p*H 5 is suspended in 10 volumes of 95% ethanol at −20°, 3–4 equiv. of iodine in 95% ethanol, per mole of mercury dimer, are added to the

(14) This reaction has been reported to occur in the lipid extraction of albumin by absolute methanol.[15]

(15) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(16) An alternative and perhaps more probable explanation, suggested to the author by Dr. W. L. Hughes, Jr., is that a chain reaction occurs between protein –SH and –S–S– linkages, giving rise to new intermolecular disulfide bonds, with the simultaneous formation of new sulfhydryl groups. This is the mechanism which has been postulated by C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 592 (1951), to explain their findings concerning the formation of albumin gels in the presence of urea.

(17) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).

(18) Further studies on the reduction of the disulfide groups in the presence of cysteine or thioglycolate have been carried out by E. Katchalski and G. Benjamin in this Laboratory (see G. Benjamin, Senior Honors Thesis in Biochemical Sciences, Harvard University, 1952). Extensive reduction can be brought about in more alkaline solutions than those employed here, but under the conditions employed in the present investigations the reduction proceeds only to the very limited extent reported here.

suspension. The mixture is centrifuged after having stood several hours, with frequent stirring. The paste is resuspended in 40% ethanol, containing 5×10^{-4} M cysteine (or dialyzed against a buffer containing cysteine at this concentration), and finally dialyzed against a convenient buffer in order to render the preparation alcohol- and salt-free.

Acknowledgments.—I am indebted to the late Professor E. J. Cohn and to Professors J. L. Oncley and J. T. Edsall for helpful discussion and advice; and especially to Dr. W. L. Hughes, Jr., whose constant guidance and suggestions have been indispensable in carrying out this research.

BOSTON, MASSACHUSETTS

[CONTRIBUTION NO. 1870 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of L-Tyrosinhydroxamide in Aqueous Solutions at 25° and *p*H 6.9[1]

BY ROBERT J. FOSTER, ROBERT R. JENNINGS AND CARL NIEMANN[2]

RECEIVED JANUARY 4, 1954

The optimum *p*H for the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide in aqueous solutions at 25° has been found to be 6.95 ± 0.05. For the system α-chymotrypsin–L-tyrosinhydroxamide, in aqueous solutions 0.2 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, at 25° and *p*H 6.9, the value of $K_S = 41 \pm 2 \times 10^{-3}$ M and the value of $k_3 = 3.6 \pm 0.2 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. D-Tyrosinhydroxamide has been found to function as a competitive inhibitor in the above system and under the above conditions $K_I = 40 \pm 8 \times 10^{-3}$ M. An explanation is offered to account for the fact that the *p*H optimum for the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide lies in a more acid region than that of acetyl-L-tyrosinhydroxamide, or of acetyl-L-tyrosinamide, and for the fact that the value of k_3 for L-tyrosinhydroxamide is substantially less than that of acetyl-L-tyrosinhydroxamide at their respective *p*H optima.

The α-chymotrypsin catalyzed hydrolysis of L-phenylalaninamide, and of L-tyrosinamide, in aqueous solutions at 25° and *p*H 7.8, was first reported by Fruton and Bergmann[3] in 1942, and seven years later Kaufman and Neurath[4] found that L-tyrosine ethyl ester, in 30% aqueous methanol solutions at 25° and *p*H 7.8, was also slowly hydrolyzed in the presence of the same enzyme. In the following year Balls and his co-workers[5,6] noted that the apparent rate of the α-chymotrypsin catalyzed hydrolysis of L-tyrosine ethyl ester, in aqueous solutions at 25°, was approximately seven times faster at *p*H 6.25 than at *p*H 7.8, and that the optimum *p*H for this system was 6.25. At about the same time the Goldenbergs[7] reported that the apparent rate of the α-chymotrypsin catalyzed hydrolysis of L-phenylalanine ethyl ester, in aqueous solutions at 25°, was approximately nine times faster at *p*H 6.4 than at *p*H 7.85 and that the optimum *p*H for this system was 6.4 when determined with one analytical procedure and approximately 6.5 when determined with another. In a second communication[8] it was noted that the optimum *p*H for the α-chymotrypsin catalyzed hydrolysis of L-leucine ethyl ester, in aqueous solutions at 25°, also appeared to be dependent upon the analytical procedure used being 6.8 in one instance and 7.2–7.3 in another.

While there is no doubt that the optimum *p*H for the α-chymotrypsin catalyzed hydrolysis of L-tyrosine ethyl ester, in aqueous solutions at 25°, lies in a lower *p*H region than that observed for the comparable hydrolysis of acetyl-L-tyrosine ethyl ester[6] it is clear that there is need for considerably more data relative to the behavior of specific substrates containing α-amino, or α-ammonium, groups than is now available.

Since the hydrolysis of hydroxamides can be followed by a convenient and precise colorimetric procedure[9] and because data are available with respect to the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide in aqueous solutions at 25° and *p*H 7.6,[10,11] *i.e.*, the optimum *p*H for this system, we have, in this instance, determined the kinetics of the α-chymotrypsin catalyzed hydrolysis of L-tyrosinhydroxamide in aqueous solutions 0.2 M with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer and at 25° and the optimum *p*H for this system. In the determination of the optimum *p*H of the above system two different types of buffers were employed, one, a 0.1 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer and the other, a cacodylic acid–sodium cacodylate buffer 0.1 M in arsenic introduced as cacodylic acid.

It will be seen from the data presented in Fig. 1 that the dependence of the relative activity of the system under investigation upon *p*H can be represented by a fairly symmetrical curve with a maximum in the region of *p*H 6.9–7.0. Thus, in contrast to L-tyrosine ethyl ester and acetyl-L-tyrosine ethyl ester, where the difference in their respective *p*H optima is reported to be approximately 1.6 *p*H units,[6] the difference in the *p*H optima of L-tyrosin-

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