formation of a stable product required that the phosphorus oxychloride and phosphorus trichloride reactions occur at comparable rates. When the phosphorus trichloride reaction predominated, carbon was formed spontaneously; when the phosphorus oxychloride reaction predominated, the product exhibited instability through the elimination of hydrogen chloride. At least one carbon-bound hydrogen atom had to be replaced by chlorine if the loss of hydrogen chloride was to be blocked.

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[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

Action of Sulfating Agents on Proteins and Model Substances. I. Concentrated Sulfuric Acid

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The method of preparation, properties and possible practical applications of a derivative of wheat gluten obtained by the action of cold, concentrated sulfuric acid have recently been described.[2] The sodium, potassium and ammonium salts of this derivative possess the unique property of absorbing almost immediately 100–300 times their weight of cold water to give stiff gels. Because information that might lead to an understanding of this property would be of theoretical and practical value, a study has been made to determine the specific groups in proteins that react with sulfuric acid.

The effect of sulfuric acid on proteins has not previously been investigated in detail. Uchino[3] kept silk fibroin in contact with concentrated sulfuric acid at 7–8° for an extended period of time and followed the extent of hydrolysis by amino nitrogen determinations. There appeared to be no measurable hydrolytic changes until after the twelfth day. Harris, Mease and Rutherford[4] treated wool with various concentrations of sulfuric acid, observed that sulfate was bound non-ionically, and suggested that combination occurred with the amino groups. However, evidence obtained in the present study indicates that the amino groups are not involved. Instead, the bound sulfate can be accounted for almost quantitatively as acid sulfate esters of the hydroxyl groups of the β-hydroxyamino acids and of hydroxyproline. In addition, a small portion of the bound sulfate is accounted for by the reaction of sulfuric acid with sulfhydryl groups to form thiosulfates, and with part of the phenolic rings of tyrosine to form sulfuric acid esters or sulfonic acids in varying proportions, depending upon the conditions of the reaction.

The participation of the hydroxyl groups of the aliphatic hydroxyamino acids is in accord with the known reaction of sulfuric acid with alcohols to form alkyl acid sulfates.[5] Since the amino and many other polar groups of proteins do not react, sulfuric acid appears to be more selective in reacting with the aliphatic hydroxyl groups of proteins than other reagents so far investigated.[6] In contrast to this selectivity, another sulfating agent, the addition product of pyridine and chlorosulfonic acid, reacts with most types of polar groups in proteins, as will be demonstrated in a subsequent paper.[7]

In general the reaction was performed by mixing proteins or model substances with concentrated sulfuric acid at temperatures below 0°. The mixtures were allowed to warm to room temperature, poured over ice, brought to neutrality with sodium hydroxide, dialyzed to remove inorganic sulfates and dried from the frozen state. Yields varied from 100 to 130% of the weight of the original material. The products were analyzed for nitrogen, bound sulfate and, in some cases, for total sulfur, amino nitrogen and tyrosine. With materials that could not be isolated by dialysis, the extent of reaction was measured, in solutions from which excess sulfuric acid had been removed as barium sulfate, by determining, after acid hydrolysis, the ratio of sulfate sulfur to nitrogen.

Since serine, threonine, hydroxyproline and cysteine were found to react more slowly than proteins, they were mixed with sulfuric acid at room temperature and allowed to stand for varying lengths of time before continuing with the analyses.

Aliphatic Hydroxyl Groups—The amounts of sulfate bound by a series of proteins are compared in Table I with their contents of aliphatic hydroxyamino acids. The materials used varied

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Not copyrighted.

(2) Reitz, Ferrel, and Olcott, *Ind. Eng. Chem.*, **36**, 1149 (1944).

(3) Uchino, *J. Biochem.* (Japan), **20**, 65 (1934).

(4) Harris, Mease and Rutherford, *J. Res. Natl. Bur. Stand.*, **18**, 343 (1937).

(5) Suter, "Organic Chemistry of Sulfur," John Wiley and Sons, Inc., New York, N. Y., 1945, Chap. I.

(6) Gordon, Martin and Synge, *Biochem. J.*, **37**, 538 (1943), reported that treatment of wool with methyl sulfate in alkaline solution caused partial methylation of the aliphatic hydroxyl and the phenolic groups. Blackburn and Phillips, *ibid.*, **38**, 171 (1944), found that methylation with this reagent in neutral or acid solution also introduced some bound sulfate into wool and suggested that the reaction involved the hydroxyl groups of hydroxyamino acids and proximal "activated" peptide bonds, as well as carboxyl and other groups.

(7) Reitz, Ferrel, Olcott and Fraenkel-Conrat, THIS JOURNAL, **68**, 1031 (1946).

in β-hydroxyamino acid content from sericin, which contained 38% (calculated as serine), to synthetic polypeptides which contained none. With few exceptions the order of decreasing sulfate content was the same as that of decreasing aliphatic hydroxyamino acid content.[8] That the hydroxyl groups in the side chains of hydroxyproline were as available for esterification as were those of the β-hydroxyamino acids was indicated by the considerable amounts of sulfate bound by gelatin and isinglass which are reported to contain 14.4%[9] and 4.7%[10] of hydroxyproline, respectively. The excess of sulfate bound over β-hydroxyamino acids present in some of the proteins listed in Table I may be due to the unknown amounts of hydroxyproline present. No reliable method for the quantitative determination of this amino acid appears to be available. Attempts to use the McFarlane-Guest procedure[11] were unsatisfactory.

The successful preparation of the O-sulfuric acid esters of hydroxyproline, serine and threonine by reaction of the amino acids with sulfuric acid is further evidence for the reactivity of the hydroxyl groups in proteins.[12]

Sulfhydryl Groups.—Evidence that the sulfhydryl group is capable of reacting with concentrated sulfuric acid was obtained with cysteine from which the corresponding sulfonic acid was synthesized. $HOOC—CHNH_2—CH_2SH + H_2SO_4 \rightarrow HOOC—CHNH_2—CH_2—S—SO_3H + H_2O$. Micheel and Emde[13] previously had prepared this compound from cystine and sulfurous acid. $HOOC—CHNH_2—CH_2SSCH_2—CHNH_2—COOH + H_2SO_3 \rightarrow HOOC—CHNH_2—CH_2—S—SO_3H + HOOC—CHNH_2—CH_2SH$. Since most proteins contain less than 1% of cysteine,[14] the reaction with the sulfhydryl group can account for only a small portion of the bound sulfate. After reaction with sulfuric acid, egg albumin no longer contained sulfhydryl groups as indicated by negative tests in 6 *M* urea solution with nitroprusside or with the Folin uric acid reagent.

The disulfide linkage does not appear to be affected by sulfuric acid in proteins[4] or in the amino acid, cystine (Table II).

Tyrosine.—When phenols are treated with cold sulfuric acid, both acid sulfates and sulfonates

TABLE I

COMPARISON OF SULFATE SULFUR INTRODUCED BY SULFURIC ACID, AND ALIPHATIC HYDROXYAMINO ACID CONTENT OF PROTEINS AND RELATED COMPOUNDS[a]

	Sulfate sulfur, %	Nitrogen, %	Per 10^4 g. of original material: Sulfate sulfur introduced,[b] g.-mole	Per 10^4 g. of original material: Hydroxyamino acids,[c] g.-mole
Sericin	7.7	9.8	37.7	37.1[f]
γ-Globulin	4.5	11.5	17.3	16.8
Gelatin	3.9	12.5	15.2	14.2[e]
Chicken feathers	4.4	11.2	18.0	14.0
Silk fibroin	4.1	12.8	16.9	12.8
Wool	4.0	12.3	15.0	12.0
Fibrin	3.5	13.1	12.6	10.5[e]
Isinglass	3.7	13.8	13.7	10.2
Egg albumin[d]	3.7	12.4	13.6	10.0
Cattle hoof	3.6	12.9	13.1	9.6
Casein	2.7	11.5	10.5	9.4[e]
Zein	2.7	13.2	9.7	7.4[e]
β-Lactoglobulin	2.4	12.9	8.6	6.3
Insulin	2.2	12.4	7.9	6.1
Glutenin[d]	2.6	12.2	9.3	6.1
Wheat gluten[d]	2.4	13.7	7.5	5.6
Gramicidin	1.3	10.4	5.9	4.8
Gliadin	2.3	14.6	7.9	4.6
Protamine sulfate	1.9	..	6.4	4.3
Polyglutamic acid	0.4	7.6	1.6	0.5[e]
Polyglutamine	0.2	18.5	0.7	..
Nylon	0.2	10.4	0.5	..
Polyglycine	0.2	..	0.5	..

[a] All analyses on air-dry basis. [b] Calculated as described in footnote 31. [c] The analytical values for total-β-hydroxyamino acids are probably low by approximately 10%.[39] For gelatin, isinglass, casein, and zein, the values of 9.5, 3.4, 1.5 and 0.6 equivalents, respectively, have been added for the hydroxyproline contents, as reported in the literature and corrected for the moisture content of our preparations.[9,10,41] Information concerning the hydroxyproline contents of the other proteins is scanty. It is possible that some of the discrepancies between bound sulfate sulfur and aliphatic hydroxyamino acid contents may be due to the unknown quantities of hydroxyproline present. For the role of carbohydrates, see footnote 8. [d] The values are based on the weighted averages of the sulfate sulfur contents of the soluble and insoluble fractions. All other proteins used, with the exception of wool, were mostly water-soluble after sulfation. For gluten the sulfate sulfur contents of the soluble and insoluble portions were 2.3% and 2.6%, respectively. The gel portion represented 43% of the total yield. [e] Similar amounts of apparent hydroxyamino acids were determinable in samples of pure glycine by the methods used, hence this figure is at the lower limit of accuracy of the method. [f] The value of 33.0 was also obtained. See footnote 37.

(8) The reaction of cold sulfuric acid on other high polymers containing hydroxyl groups was demonstrated with polyvinyl alcohol and starch, which were found to contain 17.5 and 12.1%, respectively, of bound sulfate sulfur after isolation of the reaction products. The presence of carbohydrate in proteins, either as a difficultly removable impurity (wheat gluten) or as an integral part of the molecule (egg albumin) might be expected to account for part of the sulfate bound through treatment with sulfuric acid. However, the sulfated derivatives of wheat gluten and egg albumin were found to contain less than 1% carbohydrate compared to the 10% and 3% originally present, as determined by the orcinol method of Tillmans and Philippi (*Biochem. Z.*, **215**, 36 (1929)). On the other hand, by the same test, sulfated ovomucoid was found to contain approximately 75% of the carbohydrate present in the untreated protein, and also considerably more sulfate sulfur (17.5 moles per 10^4 g. original protein) than could be accounted for on the basis of its β-hydroxyamino acid content (7.6 moles). Many of the proteins listed in Table I are known to be free from, or to contain only traces of, carbohydrates (Sørenson and Haugaard, *Biochem. Z.*, **260**, 247 (1943)).

(9) Bergmann, *J. Biol. Chem.*, **110**, 471 (1935).

(10) Beveridge and Lucas, *ibid.*, **155**, 547 (1944).

(11) McFarlane and Guest, *Can. J. Res.*, **17B**, 139 (1939).

(12) The preparation of the O-acid sulfates of serine, threonine, and hydroxyproline by reaction with sulfuric acid is analogous to the synthesis of the O-acid phosphates of serine and hydroxyproline by reaction with phosphoric acid and phosphoric anhydride as described by Levene and Schormüller, *J. Biol. Chem.*, **105**, 547 (1934); **106**, 595 (1934); and Plimmer, *Biochem. J.*, **35**, 461 (1941).

(13) Micheel and Emde, *Z. physiol. Chem.*, **265**, 266 (1940).

(14) Hess and Sullivan, *J. Biol. Chem.*, **151**, 635 (1943).

result, in proportions which vary with the conditions employed.[5,15] Under more drastic conditions, only sulfonates are obtained. Tyrosine and protein phenolic groups appear to behave similarly, although the results with proteins are difficult to interpret quantitatively due to the complexity of the reaction.

Tyrosine reacted with sulfuric acid under the conditions usually used with proteins. Total and sulfate sulfur analyses indicated that the product was 25% sulfate and 63% sulfonate. However, after contact with sulfuric acid for 1 hour at room temperature, only traces of sulfate remained[16] (Table II).

TABLE II

EFFECT OF CONCENTRATED SULFURIC ACID ON AMINO ACIDS[a]

Amino acid[b]	Equivalents of sulfate sulfur bound[c]	Equivalents of total sulfur bound[c]
Serine	1.02	..
Threonine	0.95	..
Hydroxyproline	1.02	..
Cysteine	0.82	..
Tyrosine[d]	.05	0.95
Tryptophan	.04	.17
Phenylalanine	.01.	.04
Histidine	.01	.01
Cystine	.04	..

[a] One hour at room temperature (23–25°). [b] In addition to those listed, a mixture containing arginine, tyrosine, glutamic acid, lysine, histidine, cystine, tryptophan and aspartic acid was found to contain an amount of sulfate sulfur corresponding to 0.013 mole per mole of nitrogen after reaction with sulfuric acid. [c] Based upon sulfur and nitrogen analyses of aliquots of the same solutions. [d] For the effect of milder conditions of reaction see the discussion of tyrosine in the experimental section.

The type of reaction undergone by protein tyrosine groups was investigated by means of tyrosine colorimetric measurements and also by a comparison of total and sulfate sulfur contents. Control experiments with the Folin phenol reagent showed that both phenolic sulfates and sulfonates had only 10–25% of the chromogenic value of the untreated compounds and that only in the case of the sulfates could the original chromogenic value be regenerated by acid hydrolysis. The apparent tyrosine content of intact sulfated proteins was less than that of the original proteins, suggesting that some sulfation or sulfonation had occurred (Table III). After acid hydrolysis, only part of the chromogenic value was regained, hence only part of the reacted tyrosine had been in the form of sulfate, and the remainder, sulfonate. When insulin was treated with sulfuric acid under very mild conditions, the product contained almost as much apparent tyrosine after hydrolysis as did the original protein, indicating that the tyrosine that had reacted was almost entirely in the form of sulfate.

TABLE III

EFFECT OF SULFURIC ACID ON THE APPARENT TYROSINE CONTENT OF PROTEINS[a]

Protein	Treatment	Apparent tyrosine content: Before hydrolysis,[b] %	Apparent tyrosine content: After hydrolysis, %
Egg albumin	None	3.1	4.4
Egg albumin	−18° to +23°, 30 minutes	2.7	2.7
β-Lactoglobulin	None	2.9	4.5
β-Lactoglobulin	−18° to +23°, 30 minutes	2.0	2.9
Insulin	None	8.9	11.3
Insulin	−18°, 10 minutes	5.7	11.0
Insulin	−18° to +23°, 30 minutes	4.7	8.3
Silk fibroin	None	...	11.5
Silk fibroin	−18°, 20 minutes	...	8.9
Silk fibroin	−18° to +23°, 30 minutes	...	4.8
Silk fibroin	23°, 1 week	...	1.7

[a] Determined colorimetrically according to Herriot with the Folin reagent. Air-dry basis. [b] As previously stated (Fraenkel-Conrat and Cooper, *J. Biol. Chem.*, **154**, 227, (1944)), the standard curve for intact proteins differed from that for free tyrosine or protein hydrolyzates. Thus the tyrosine values for intact egg albumin and β-lactoglobulin ranged from 3.5–2.6% for 5–20 mg. samples; for insulin values of 10.0–7.6% were obtained with 1.5–6 mg. samples. The values listed for unhydrolyzed proteins are therefore only rough approximations. Several investigators (see Miller, *J. Biol. Chem.*, **146**, 345 (1945)) have reported that the apparent tyrosine content of intact proteins is considerably smaller than that found after hydrolysis, without reference to differences in the curves obtained with intact and hydrolyzed proteins.

Proteins which were allowed to stand in sulfuric acid at room temperature for increasing lengths of time contained increasing amounts of tyrosine sulfonate as indicated by low apparent tyrosine contents, not increased by hydrolysis, and by a relative increase in total sulfur compared to the sulfate sulfur content (Table IV).

Other Protein Groups.—The amino groups of proteins or amino acids do not react with sulfuric acid. Sulfated proteins contained amino nitrogen equivalent to or slightly higher than that of the original protein. The amino nitrogen content of egg albumin was 0.64%, that of the sulfated derivative, 0.63% corrected to the original weight. On the same basis, these values were 1.13 and 1.26% for β-lactoglobulin, 0.18 and 0.19% for gluten, 0.10 and 0.35% for silk fibroin and 0.57 and 0.80% for gelatin. Control experiments[7] showed that alkyl sulfamates, if they had formed, would not yield amino nitrogen under the conditions of the Van Slyke analysis. The nonreactivity of the amino groups in amino acids is indicated by the results shown in Table II.

Harris, Mease and Rutherford[4] noted that the base-binding capacity of wool was greatly en-

(15) Haworth and Lapworth, *J. Chem. Soc.*, 1299 (1924).

(16) Conklin and Johnson, THIS JOURNAL, **54**, 2914 (1932), prepared tyrosine monosulfonic acid from tyrosine and hot concentrated sulfuric acid.

hanced upon treatment with 80–95% sulfuric acid, while the acid-binding capacity was decreased, and concluded that the amino groups had been transformed to sulfamate groups: $RNH_2 + H_2SO_4 \rightarrow RNHSO_3H + H_2O$. In the present study, attempted analyses by a dye-binding technique[17] showed an absence of basic groups, even though the original number of free amino groups was demonstrable by the Van Slyke method. A similar behavior has been noted with other materials containing many carboxyl groups such as pepsin and polyglutamic acid,[18] and may be ascribed to the fact that the basic groups of these very acid materials do not carry a charge at the *p*H 2.2 used for these analyses. The decrease in acid-binding capacity of sulfated wool observed by Harris, *et al.*, may be explained similarly.

Evidence that the guanidyl groups in proteins do not react with sulfuric acid was obtained with the use of protamine sulfate, in which 68% of the nitrogen is guanidyl nitrogen. Only small amounts of bound sulfate, approximately equivalent to the amount of serine known to be present,[19] were introduced (Table I). Arginine bound no sulfate under the more severe conditions used with amino acids.

The non-participation of the amide groups was suggested by the relatively small amounts of sulfate bound by gliadin and gluten, in which 20–25% of the total nitrogen is amide nitrogen, and demonstrated by the trace amounts bound by asparagine and by the polyamide of polyglutamic acid in which all of the polar groups are primary amides (Table I).

That sulfuric acid did not combine with the peptide linkage under the usual conditions of sulfation was indicated by the comparatively small number of sulfate radicals introduced into many proteins, and particularly by the very small amounts introduced into model substances such as nylon, polyglycine, and polyglutamic acid and its polyamide.

Tryptophan and phenylalanine bound only traces of sulfate sulfur but measurably more total sulfur (Table II). Presumably this is evidence of nuclear substitution, but on a much smaller scale than is observed with tyrosine. After sulfation by the usual technique, gramicidin was found to contain 1.35% sulfate sulfur compared to 1.54% total sulfur.[20] The difference is believed to be due to sulfonation of the indole ring. Since gramicidin contains approximately 40% tryptophan,[21] the indole nuclei of proteins are probably only slightly involved in the reaction with sulfuric acid.

The tryptophan present in many proteins is known to be destroyed during acid hydrolysis. It therefore appeared of interest to determine whether contact with cold sulfuric acid affected the integrity of the indole ring. Since, with both gramicidin (after hydrolysis) and tryptophan, at least 75% of the original chromogenic value was still present after the usual sulfuric acid treatment, it was concluded that no extensive degradative changes of the indole nuclei had occurred.

The ring system of histidine did not bind sulfur either as sulfate or sulfonate (Table II).

Effect of Prolonged Treatment with Sulfuric Acid.—Uchino,[3] by amino nitrogen analyses, found silk fibroin stable to sulfuric acid for eleven days at 7–8°, after which a slow liberation of amino groups occurred. In the present work, a sample of γ-globulin in sulfuric acid was held for ten days at 4° (desiccator). Amino nitrogen analyses of the product indicated that hydrolysis of approximately 2–3% of the peptide linkages had occurred. However, when held at room temperature (desiccator) in sulfuric acid for periods up to one week, proteins were found to be degraded to a considerable extent, as indicated by high amino nitrogen values and by the fact that the amount of material that could be recovered after dialysis

TABLE IV

EFFECT OF STANDING IN SULFURIC ACID AT ROOM TEMPERATURE ON THE BOUND SULFATE SULFUR, TOTAL SULFUR AND HYDROXYAMINO ACID CONTENTS OF PROTEINS

Protein	Time of contact, hr.	Yield,[a] %	Sulfate sulfur, %	Total sulfur,[b] %	β-Hydroxy-amino acid moles/10 g.
Sericin	0.3[c]	135	8.6	...	31
Sericin	0.5[d]	144	7.7	8.6	31
Sericin	24	72	4.5	5.5	..
Sericin	48	46	2.2	3.1	22
Sericin	192[e]	18	0.6	3.0	18
Silk fibroin	0.5[d]	115	4.1	4.9	13
Silk fibroin	48	68	0.8	2.3	11
Gelatin	0.5[d]	122	4.1	4.1	..
Gelatin	192	26	3.0	3.1	..
Gliadin	0.5[d]	101	2.3	4.3	4.0
Gliadin	192[e,f]	39	0.2	3.1	4.5
Insulin	0.3[c]	110	2.5	...	..
Insulin	0.5[d]	...	2.2	...	..

[a] After dialysis and lyophilization, based upon weight of protein used. [b] The sulfur contents of proteins used were: sericin, 0.34%; silk fibroin, 0.08%; gliadin, 1.1%; and gelatin, 0.32%. [c] Held at −17° for twenty minutes. [d] Time to warm to room temperature. [e] Treated with 103% sulfuric acid. Sericin sulfated with concentrated sulfuric acid contained 0.9%. [f] When a similar preparation was neutralized with 28% ammonium hydroxide solution at −5 to −15° before dilution with water, the yield (after dialysis) was 84%, but the sulfate and total sulfur contents were the same as in the product diluted in the usual manner. When a similar preparation was isolated by precipitation and repeated washings with ether and acetone, the product contained 5.5% sulfate sulfur. The alkali needed to bring this product to *p*H 7.1 indicated the liberation of 94% of its sulfate sulfur as inorganic sulfate. After dialysis, 77% of the nitrogen was recovered. The product then contained only 0.35% sulfate sulfur and 0.40% amino nitrogen.

(17) Fraenkel-Conrat and Cooper, *J. Biol. Chem.*, **154**, 239 (1944).

(18) Fraenkel-Conrat and Olcott, *ibid.*, **161**, 259 (1945).

(19) Block and Bolling, *Arch. Biochem.*, **6**, 419 (1945).

(20) The sulfate sulfur introduced can be accounted for by the hydroxyl groups present. The source of the hydroxyl groups has not been definitely established, but ethanolamine is liberated during acid hydrolysis (Synge, *Biochem. J.*, **39**, 355 (1945)).

(21) Hotchkiss, *Adv. in Enzym.*, **4**, 153 (1943).

decreased progressively, to as low as 10%. This breakdown of the peptide chain could be averted if the reaction product was isolated in such a manner as to minimize or avoid contact with aqueous acid. This was achieved either by neutralizing a gliadin-containing reaction mixture at −5 to −15° with concentrated ammonium hydroxide solution, or by precipitating the product with ether and removing all free sulfuric acid by repeated washing with ether and acetone (*cf.* Table IV, footnote *f*). The latter product contained 5.5% sulfate sulfur, most of which was released upon contact with water and could be titrated as free sulfuric acid. The protein was not markedly degraded in the course of this neutralization, as indicated by amino nitrogen values and recovery after dialysis. A plausible interpretation of these findings is that prolonged contact with sulfuric acid leads to activation of peptide bonds, either through sulfation of the enol form or some other mechanism. In the course of the usual isolation procedure many of these peptide bonds are presumably split concomitant with the liberation of the sulfate, whereas the modified procedures lead to the liberation of most of the sulfate without breakdown of the corresponding peptide bond.

Independent of this effect on the peptide chains of proteins prolonged sulfation at room temperature was observed to cause a marked loss in the amounts of sulfate bound by the hydroxyl groups (Table IV). This was evident with all proteins studied, and independent of the conditions of isolation of the derivatives. In attempts to exclude hydrolysis as a factor responsible for this loss of sulfate, several proteins were treated for several days with sulfuric acid containing excess sulfur trioxide (103% sulfuric acid). However, the resultant products were as low in bound sulfate as were those treated with concentrated sulfuric acid (Table IV, footnote *e*).

In order to determine whether the loss of bound sulfate in proteins was connected with destruction of the β-hydroxyamino acids, these were determined in several sulfated derivatives. With egg albumin and silk fibroin, there was no loss of β-hydroxyamino acids. However, sericin, the protein richest in serine, differed from these in that there was a loss of approximately one-third of the total β-hydroxyamino acids after the protein had been in contact with sulfuric acid for one week at room temperature. This was observed both with dialyzed preparations and diluted reaction mixtures, hence the possibility was excluded that a fraction rich in hydroxyamino acids might have been lost selectively during dialysis. If the destruction of serine had been due to dehydration to dehydroalanine, there would have been a corresponding increase in the ammonia content of the hydrolysate. However, no appreciable increase was found. These observations could be explained as being the result of the formation of ether linkages or cyclic neutral sulfates involving proximal serine residues, but no direct evidence for such reactions has been obtained.

In contrast to the instability of the sulfate ester groups of proteins in concentrated sulfuric acid, serine and threonine acid sulfates appeared to be comparatively stable. The ratio of bound sulfate sulfur to nitrogen for serine had decreased from 1.0 to 0.93 after treatment for one week at room temperature. Threonine, after four weeks, had a sulfur to nitrogen ratio of 0.75.

Stability of Sulfate Esters in Acids and Alkalies.—It appeared of interest to determine the relative stabilities of the sulfate esters of amino acids in 1 *N* acid and alkali. Such solutions were permitted to stand for one day, then brought to neutrality and tested for the presence of sulfate ions by the addition of barium chloride. The sulfates of serine, threonine, and hydroxyproline were stable in acid under these conditions and that of hydroxyproline was also stable in the presence of alkali.[22] Serine and threonine acid sulfates were about 10% hydrolyzed by 1 *N* sodium hydroxide in one day. No hydrolysis could be detected in 0.5% pyridine.

Sericin and gelatin sulfates were exposed to buffers at *p*H 2.2 and 11.5 for one day at room temperature, and subsequently dialyzed against 0.5% pyridine to remove free sulfate ions.[23] They were found to have lost almost no sulfate at *p*H 2.2 but 58 and 31% of their sulfate sulfur contents, respectively, at *p*H 11.5. Since sericin is very rich in serine and gelatin contains relatively more hydroxyproline, these data are confirmatory evidence for the different labilities in dilute alkali of serine and hydroxyproline sulfates.

Determinations of total acid groups in sulfated proteins by the dye technique[17,18] were found to lack quantitative validity because of the partial hydrolysis of the sulfate bonds at *p*H 11.5; however, it was possible to demonstrate the presence of many more acid groups than were present in the original protein.

Experimental

Materials.—Wheat gluten, zein, gelatin and nylon molding powder were commercial products. Crystalline egg albumin and β-lactoglobulin were prepared by the method of Kekwick and Cannan,[24] and Palmer,[25] respectively. The wool and feathers had been washed with water and then extracted with benzene. Cattle hoofs were washed, then dried in an oven at 70°, and pulverized in an iron mortar. All keratins were subsequently ground in a Wiley mill. Sericin was prepared by the method of Rutherford

(22) Rollins and Calderwood, THIS JOURNAL, **60**, 2312 (1938), described the marked stability toward hydrolysis of aminoethylsulfuric acid.

(23) A sample of sulfated sericin of original sulfate content, 7.8%, contained 7.5% after further dialysis for 9 days against repeated changes of distilled water; 7.3% after 9 days against repeated changes of 0.5% pyridine. Barritt, Bowen, Goodall and Whitehead, *Analyst*, **63**, 782 (1938), reported that ionically bound sulfate could be quantitatively removed from wool by extraction with this solvent.

(24) Kekwick and Cannan, *Biochem. J.*, **30**, 227 (1936).

(25) Palmer, *J. Biol. Chem.*, **104**, 358 (1934).

and Harris.[26] The silk fibroin preparation was that portion of the silk remaining after four successive treatments with hot water. Glutenin and gliadin were prepared by an unpublished method of fractional precipitation from dilute acid solution with alkali. Polyglutamic acid was obtained from a culture of a particular strain of *Bacillus brevis* by the method of Bovarnick.[27] The polyamide was synthesized from the peptide and has been characterized previously.[28] The polyglycine preparation was also previously described.[29] Casein was obtained by precipitation from skim milk at *p*H 4.6, dissolved in dilute alkali, and reprecipitated. Bovine fibrin was obtained from fresh beef blood by whipping. Protamine sulfate and insulin were kindly furnished by Eli Lilly and Company, γ-globulin by the Cutter Laboratories, isinglass by Connaught Laboratories, gramicidin by the Wallerstein Company, and ovomucoid by E. F. Jansen, of this Laboratory. Most of the proteins were finely ground, usually to pass a 100-mesh sieve. Wool and silk fibroin were ground to pass a 60-mesh sieve. The amino acids were commercial products of high purity.

Sulfation Procedure.—Most of the materials listed in Table I were treated as follows: One gram was introduced into a two-ounce screw-cap bottle and 2 to 4 ml. of absolute alcohol was added to prevent subsequent lumping.[30] The bottle was closed to the air and placed in a cold room at −34°; 20 ml. of concentrated sulfuric acid contained in a stoppered graduate was cooled similarly. When the protein and acid had reached −34°, the protein was suspended in the alcohol and the sulfuric acid added with stirring. The mixture was then allowed to stand at room temperature with occasional stirring until the temperature reached 23°. This usually required about thirty minutes. The reaction mixture was then poured over about 400 ml. of cracked ice, and brought to *p*H 7.5 with 10 *N* sodium hydroxide. Additional ice was supplied as needed to maintain the temperature below 15°. Toluene was added as a preservative and the solution was dialyzed against running tap water for two days and then against distilled water until the dialysate showed a negative test for sulfate. Usually three changes of distilled water were sufficient.[23] Any insoluble portion was separated and dried with acetone and ether. The soluble portion was lyophilized after being concentrated to small volume in Cellophane tubes hung in a current of warm air.[31]

With compounds of low molecular weight, which could not be isolated by dialysis, the extent of the reaction was measured in the following manner: Free sulfate ions were removed by the addition of barium chloride, or barium carbonate as described by Bordwell, Suter and Weber.[32] The barium sulfate or mixture of barium sulfate and barium carbonate was carefully washed and the washings added to the original solutions. One aliquot of the combined filtrates was analyzed for nitrogen, and a second aliquot was analyzed for bound sulfate by a modification of the Mease method[33] (see below). The nitrogen analysis served as a method of determining the amounts of the original substance or its derivative recovered, and the sulfur to nitrogen ratio served as an indication of the amount of sulfate sulfur that had been introduced in the compounds.

Analytical Methods.—Nitrogen was determined by the Kjeldahl procedure. Total sulfur was determined by means of the Parr bomb.

For sulfate sulfur determinations the Mease method[33] was applied as follows. The sample, containing in the order of 10 mg. of sulfur, was hydrolyzed in approximately 7 ml. of 6 *N* hydrochloric acid in a boiling water-bath for one hour. Any insoluble material was filtered, the residue washed thoroughly with hot water, the filtrate diluted to about 150 ml., and sulfate sulfur determined as barium sulfate. Preparations which had been isolated as the barium salts were not filtered before adding excess barium chloride.

Although the Mease method was found to give satisfactory analytical data for amino acid sulfates, a few proteins were hydrolyzed by refluxing for sixteen hours, in order to determine whether the protein sulfate esters were equally labile. Sulfate sulfur was found to be slightly higher in some products. The greatest difference was observed with an insoluble fraction of gluten sulfate which had an apparent sulfate sulfur content of 2.3% by the regular technique and 2.6% after the more vigorous hydrolysis. The possibility that at least part of the additional sulfate may have arisen from secondary decomposition of sulfur-containing amino acids cannot be excluded.

Amino nitrogen was determined in the Van Slyke manometric apparatus[34]; proteins and protein derivatives were permitted to react for fifteen minutes, amino acids and their derivatives for three minutes.

Tyrosine was determined colorimetrically by Herriott's modification[35] of the Folin method. This procedure was used for proteins, both intact and after hydrolysis with boiling 6 *N* hydrochloric acid for six hours. Tryptophan was determined by the method of Horn and Jones.[36] Total acid and basic groups were determined by a dye technique.[17,18]

Total β-hydroxyamino acids were determined by the periodate method of Nicolet and Shinn[37] and of Van Slyke, *et al.*[38] It was not possible to obtain recoveries of ammonia corresponding to more than 90% of added serine or threonine; hence the results shown in Table I may be expected to be low by at least 10%.[39] For the most part the data agree fairly well with the few values recorded in the literature.[37] More accurate analyses based on the

(26) Rutherford and Harris, *J. Res. Natl. Bur. Stand.*, **24**, 415 (1940).

(27) Bovarnick, *J. Biol. Chem.*, **145**, 415 (1942).

(28) Fraenkel-Conrat, Cooper and Olcott, THIS JOURNAL, **67**, 950 (1945).

(29) Fraenkel-Conrat, Cooper and Olcott, *ibid.*, **67**, 314 (1945).

(30) Alcohol was used only with those preparations which were later to be isolated by dialysis. The presence of ethyl hydrogen sulfate, formed by the action of sulfuric acid on alcohol, otherwise would have been a complicating factor in the analyses of sulfated substances of low molecular weight.

(31) In a number of preliminary experiments, calculations were based upon the assumption that the dried protein sulfate existed in the form of the sodium salt, R—OSO_3Na. Discrepancies in the sulfate and nitrogen contents of the derivatives suggested that other cations might be involved. Analyses by spectroscopic techniques showed the presence of 3.1% calcium, 0.9% magnesium, and 0.06% sodium in a preparation of sulfated gliadin and 1.4% calcium, 0.4% magnesium, and 0.01% sodium in a preparation of sulfated egg albumin. Cation exchange apparently had taken place during dialysis against tap water. Subsequent calculations were based upon nitrogen analyses. Moles sulfate S introduced per 10^4 g. original protein =

$$\frac{\%\text{ sulfate S in sulfated derivative} \times \%\text{ N in original protein}}{32 \times \%\text{ N in sulfated derivative}} \times 100.$$

(32) Bordwell, Suter and Weber, THIS JOURNAL, **67**, 30 (1945).

(33) Mease, *J. Res. Natl. Bur. Stand.*, **13**, 617 (1934).

(34) Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).

(35) Herriott, *J. Gen. Physiol.*, **19**, 283 (1935).

(36) Horn and Jones, *J. Biol. Chem.*, **157**, 153 (1945).

(37) Nicolet and Shinn, *J. Biol. Chem.*, **142**, 139 (1942). The value reported in Table I for the hydroxyamino acid content of sericin is in good agreement with that very kindly determined by Nicolet and Shinn (personal communication), on a sample of our sericin (36 moles per 10^4 g.). In several determinations we obtained the value of 37.1 ± 0.5, but in previous runs and in more recent attempts to duplicate these values, we have consistently obtained values of 33.0 ± 0.5 for the same preparation.

(38) Van Slyke, Hiller and MacFadyen, *J. Biol. Chem.*, **141**, 681 (1941).

(39) The destruction of some serine during acid hydrolysis has been noted by several investigators (see Damodaran and Ramachandran, *Biochem. J.*, **35**, 22 (1941); Boyd and Logan, *J. Biol. Chem.*, **146**, 279 (1942)). Brand, Kassell and Saidel (*J. Clin. Invest.*, **23**, 437 (1944)) and Brand, Saidel, Goldwater, Kassell and Ryan (THIS JOURNAL, **67**, 524 (1945)) have added 10% to the determined value of serine in order to correct for this loss.

specific methods for serine and threonine did not appear justified for the purpose of this study.

Attempts were made to determine the hydroxyproline content of several proteins by the colorimetric method of McFarlane and Guest.[11] A reproducible standard curve was obtained with hydroxyproline, but gelatin and isinglass appeared to contain only 9.0% and 3.5% of hydroxyproline (dry basis) whereas others have reported that gelatin contains approximately 14–15%[9,11] and isinglass at least 4.7%.[10] Added hydroxyproline was recovered to the extent of 90–95%. With other proteins, however, in which no hydroxyproline could be detected, added hydroxyproline was only 50–70% recoverable. Recoveries were also erratic when hydroxyproline was added to a number of amino acids either mixed or singly. These results indicated that the method was of questionable value. The few literature values for hydroxyproline listed in Table I are not based on the colorimetric method.[40,41]

Tyrosine.—The product of the reaction of tyrosine with sulfuric acid under gentle conditions was largely tyrosine O-sulfate. The reaction was carried out as follows: 5 g. of tyrosine and 10 ml. concentrated sulfuric acid were separately cooled in Dry Ice, mixed, and kept at that temperature for fifteen minutes and at room temperature for approximately ten additional minutes. The mixture was poured over ice with rapid stirring, brought to neutrality by the addition of barium carbonate, and filtered. A small sample of the filtrate, upon acidification with hydrochloric acid and heating, gave a copious precipitate of barium sulfate. The remainder of the filtrate was dried (2 g.), and found to contain 4.2% total N, 4.2% amino N, 7.4% sulfate sulfur, and 7.8% total sulfur. Since the theoretical sulfur content of barium tyrosine sulfate is 9.7%, 80% of this product was sulfate, 5% sulfonate.

Tyrosine treated with sulfuric acid under the same conditions that were used with proteins (1 g. in 5 ml. sulfuric acid at −18°, permitted to come to room temperature) and isolated as described above, was found to be 25% the sulfate, and 63% the sulfonate derivative. After a similar reaction mixture had stood at room temperature for one hour the product contained no sulfate sulfur, but was almost completely tyrosine sulfonate.

Upon colorimetric analysis, the sulfate ester derivative described above gave 11% of the color corresponding to its tyrosine content, while tyrosine sulfonic acid prepared by the method of Conklin and Johnson[16] gave 23%. Hydrolysis with 6 *N* hydrochloric acid for six hours regenerated 85% of the chromogenic value of phenolic ester, but did not affect the sulfonic acid derivative.

Preparative

O-*dl*-Serine Acid Sulfate.—Twenty-five ml. of reagent sulfuric acid was added to 5 g. of serine and stirred until the serine was in solution. The reaction mixture was permitted to stand at room temperature in a desiccator for the remainder of one hour, and then poured into 2 liters of ether which had been cooled in a Dry Ice–acetone-bath. The ether was decanted and the residue washed 3 times with 1-liter portions of cold ether. It was then dissolved in a minimum amount of hot 90% ethanol and crystallized by the addition to turbidity of a mixture of equal parts of methyl ethyl ketone and ether. The crystalline product was filtered and dried *in vacuo*; yield 6.40 g. (73% of theory). The compound melted with decomposition at 203–205° (cor.).

Anal. Calcd. for $C_3H_7O_6NS$: C, 19.5; H, 3.8; sulfate S, 17.3; N, 7.6; amino N, 7.6. Found: C, 19.5; H, 3.8; sulfate S, 17.4; N, 7.5; amino N, 7.3.

That the sulfuric acid is bound to the hydroxyl rather than the amino group of serine was demonstrated in the following manner. A sample of serine sulfate was deaminized by treatment with sodium nitrite and glacial acetic acid in the macro-Van Slyke volumetric apparatus. The theoretical quantity of nitrogen was evolved. Most of the sulfate in the residual solution was found to be organically bound. If the sulfuric acid had been present as the sulfamate, and if this group were capable of reacting with nitrous acid, free sulfate ions would have been released. No attempt was made to isolate the β-acid sulfate of glyceric acid, which should have been the primary product of the reaction.

O-*dl*-Threonine Acid Sulfate.—Five grams of threonine was treated as described above for serine sulfate. The reaction mixture was poured into 2 liters of methyl ethyl ketone, cooled in a Dry Ice–acetone-bath. The solvent was decanted from the solid product, which was then washed 3 times with 1-liter portions of cold methyl ethyl ketone. The residue was dissolved in a minimum amount of 80% ethanol and crystallized by the procedure described for the serine derivative. The product was recrystallized three times by this method, finally washed with absolute ether and dried *in vacuo;* yield, 4.2 g. (50%); m. p. 141–143° (cor.). When permitted to come to equilibrium in contact with humid air, the compound analyzed correctly for a monohydrate.

Anal. Calcd. for $C_4H_9O_6NS \cdot H_2O$: sulfate S, 14.8; N, 6.45; amino N, 6.45; H_2O, 8.3. Found: sulfate S, 14.9; N, 6.41; amino N, 6.40; H_2O, 8.3.

S-*l*-Cysteine Sulfonate.—6.45 g. of *l*-cysteine hydrochloride was treated with 25 ml. of sulfuric acid for four hours at room temperature. The reaction mixture was poured into 1 liter of cold ether (−25°) with stirring. The residue was washed two times with 1-liter portions of cold ether, then dissolved in a minimum quantity of absolute alcohol. Crystallization was effected by the addition of ether. After the second or third crystallization an alcohol-insoluble fraction, which appeared to be cystine was present. The alcohol-soluble fraction was recrystallized as often as necessary to remove all free sulfate; m. p. 179–182° (hot stage) (Micheel and Emde[13] reported 184–185°); yield 2.7 g. (32%).

Anal. Calcd. for $C_3H_7O_5NS_2$: N, 7.0; S, 31.8; sulfate S, 15.9. Found (dry basis): N, 7.0; S, 31.5, sulfate S, 15.4.

Acknowledgment.—The authors are indebted to I. Ahnger, C. Cleaver, L. White and E. F. Potter for numerous analytical determinations. The crystallographic and optical properties of the hydroxyamino acid sulfuric acid esters have been investigated by F. E. Jones and will be reported elsewhere by him. E. J. Eastmond furnished the spectroscopic analyses.

Addendum.—Since this work was completed, Lustig and Kondritzer (*Arch. Biochem.*, **8**, 51 (1945)) have recorded the effect of concentrated sulfuric acid (twenty-four hours at 0°) on hair. No attempt was made to account for the sulfate sulfur (3.1–4.7%) introduced. The hydrolysate was shown to contain unchanged amounts of cystine, threonine and histidine. There was a 25% decrease in the arginine content and a 10% decrease in the serine content. The loss of approximately 35% of the tyrosine, 20% of the tryptophan and 10% of the phenylalanine was attributed to possible sulfonation.

Summary

The reaction of cold, concentrated sulfuric acid on proteins resulted in the approximately quantitative formation of acid sulfates of the

(40) A referee called our attention to the paper of J. Devine, *Biochem. J.*, **35**, 433 (1942). This author found only 10.4% of hydroxyproline in gelatin using the method of McFarlane and Guest. This is also in keeping with the experimental experience of the referee.

(41) Fuerth and Minnibeck, *Biochem. Z.*, **250**, 18 (1932).

hydroxyl groups of serine, threonine, and hydroxyproline residues. The sulfhydryl group of cysteine reacted to form the thiosulfate. Under the specific conditions used, the hydroxyl groups of tyrosine were not appreciably sulfated; some ring sulfonation occurred.

The O-sulfuric acid esters of serine, threonine, and hydroxyproline, and S-cysteine sulfonate were prepared by the action of concentrated sulfuric acid on the amino acids.

The stabilities of the protein and amino acid sulfate bonds in sulfuric acid and in dilute acid and alkaline solutions were determined.

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[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

Action of Sulfating Agents on Proteins and Model Substances. II. Pyridine-chlorosulfonic Acid

BY HENRY C. REITZ, ROBERT E. FERREL, HAROLD S. OLCOTT AND HEINZ FRAENKEL-CONRAT

The products of the reaction of wheat gluten with either pyridine-chlorosulfonic acid or cold concentrated sulfuric acid are of possible practical interest, since, when brought to neutrality, they bind large amounts of water to form stiff, translucent gels.[1a] Considerably more sulfate sulfur is introduced into wheat gluten with pyridine-chlorosulfonic acid (8.8%) than with sulfuric acid (2.5%). The action of the latter reagent on proteins in general has recently been shown to lead principally to the formation of acid sulfate esters of the aliphatic hydroxyl groups of serine, threonine, and hydroxyproline.[2] It was the object of the present study to ascertain the protein groups participating in the reaction with pyridine-chlorosulfonic acid.

Hatano[3] prepared partly sulfated proteins by means of pyridine-chlorosulfonic acid but did not identify the protein groups involved. Baumgarten, *et al.*,[4] investigated the reaction of various amino acids and peptides in cold aqueous alkaline solution with N-pyridiniumsulfonic acid, which they believed to be the sulfating agent in the pyridine-chlorosulfonic acid reaction product. They concluded that the phenolic groups of tyrosine were sulfated, and that the free amino groups and the imidazole rings were transformed to sulfamic acid derivatives, but that no reaction took place with the aliphatic hydroxyl, indole or guanidyl groups, nor with the peptide linkage.

The relatively large amounts of sulfate sulfur introduced into proteins by our technique could not be accounted for solely by the amino acid residues found to react by Baumgarten. This technique, which differed from that of Baumgarten, *et al.*,[4] in various respects, resembled that generally used for the sulfation of polysaccharides.[5] The reagent, freshly prepared from chlorosulfonic acid and excess pyridine, was mixed with the material to be sulfated and the mixture heated to 70–80° for several hours.[6] Under such conditions part or all of the primary amide, amino, guanidyl, thiol, indole, and aliphatic and phenolic hydroxyl groups of proteins or model substances bound sulfate sulfur in non-ionic manner. Of the polar groups that occur in proteins, only the imidazole and carboxyl groups and peptide linkages were not involved in the reaction.

Reaction of Basic Groups.—The participation of the amino groups in the reaction was indicated by the marked decreases in the amino nitrogen content (by the Van Slyke manometric method) of the sulfated proteins (Table I). This is in contrast to the unchanged, or slightly increased, content of amino nitrogen in proteins treated with cold concentrated sulfuric acid.[2] The formation of sulfamates was also demonstrated by the loss of amino nitrogen upon treatment of simple amines (Table II). Later work with isolated benzylamine sulfamate proved the validity of the Van Slyke values, since only 1% of the nitrogen was determined as amino nitrogen in a fifteen-minute reaction period. The amounts of sulfate sulfur bound by simple aliphatic amines appeared inconsistent until it was recognized that this might be due to the transient formation of disulfamates $RN(SO_3H)_2$ which are unstable in dilute acid.[7] Thus when reaction mixtures were kept alkaline during dilution there was an initial binding of more than 1 mole of sulfate sulfur per mole of nitrogen. In aliquots that were subsequently exposed to dilute acid, the sulfate sulfur–nitrogen ratio was

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(1a) Reitz, Ferrel and Olcott, *Ind. Eng. Chem.*, **36**, 1149 (1944).

(2) Reitz, Ferrel, Fraenkel-Conrat and Olcott, THIS JOURNAL, **68**, 1024 (1946).

(3) Hatano, *Biochem. Z.*, **145**, 182 (1924).

(4) Baumgarten, Marggraff and Dam...ann, *Z. physiol. Chem.*, **209**, 145 (1932).

(5) Gebauer-Fülnegg, Stevens and Dingler, *Ber.*, **61B**, 2000 (1928); Chargaff, Bancroft and Stanley-Brown, *J. Biol. Chem.*, **115**, 155 (1936); Karrer, Koenig and Usteri, *Helv. Chim. Acta*, **26**, 1296 (1943); Astrup, Galsmar and Volkert, *Acta Physiol. Scand.*, **8**, 215 (1944); and others.

(6) Suter, "Organic Chemistry of Sulfur," John Wiley and Sons, Inc., New York, N. Y., 1945, p. 7, suggests that the pyridine-chlorosulfonic acid reaction mixture used in the present study as well as in ref. 5 differs as a sulfating agent from what he calls pyridine sulfotrioxide (Baumgarten's N-pyridiniumsulfonic acid).

(7) Audrieth, Sveda, Sisler and Butler, *Chem. Rev.*, **26**, 49 (1940); Sveda, Thesis, University of Illinois, 1939.