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.*, **146**, 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.

found to be approximately 1 (Table II). The amino groups of proteins after dialysis and isolation appeared to be only in the form of monosulfamates, since globin, a protein rich in free amino groups, contained the same amount of sulfate when the reaction mixture was diluted and neutralized simultaneously as when it was neutralized after dilution.

TABLE I

EFFECT OF PYRIDINE-CHLOROSULFONIC ACID ON VARIOUS PROTEIN GROUPS

Protein	Increase in groups Equivalents per 10^4 g. of protein: Total acid[a]	Sulfate	Decrease in groups, %: Amino	Phenol + Indole (Intact protein)	Phenol + Indole (Ac'd hydrolyzate)
Egg albumin	30	30	76	89	0
β-Lactoglobulin	20	19	71	89	10
Silk fibroin	25	24	..	..	0
Silk sericin	31	53	..	..	..
Globin	17	20	73	..	4
Gelatin	21	21	83	..	..
Gliadin	49	35	..	..	0
Gluten	57	42	61	..	..

[a] The fact that the acid groups, determined according to ref. 12, fall short of the sulfate residues introduced in sericin is attributed to the lability in alkali of the acid sulfate esters of serine,[2] which is the predominant polar amino acid of this protein. The greater increase in acid groups over sulfate introduced in gliadin and gluten is not understood at present. Hydrolyses of amide groups was suspected but the sulfated products were found to contain amounts of amide nitrogen corresponding to those present in the original proteins.

TABLE II

SULFATE SULFUR INTRODUCED INTO SIMPLE COMPOUNDS THROUGH THE ACTION OF PYRIDINE-CHLOROSULFONIC ACID

	Equivalents of sulfate bound[a]
Benzylamine	1.5[b]
Glycine ethyl ester hydrochloride	1.8[b]
Ethanolamine	1.9
Methylguanidine sulfate	0.9
Acetyl-*l*-histidine monohydrochloride	0.1
N-acetyl-*l*-tyrosine	1.0
N-lauroyl-*l*-tyrosine	0.9
Indole-3-acetic acid	1.0
Acetyl-*d,l*-tryptophan	1.0
Adipamide	1.0
Heptamide	0.9

[a] Based upon the ratio of sulfur to nitrogen. Recoveries of nitrogen were at least 80% for all compounds except N-lauroyl-*l*-tyrosine (57%). The errors inherent in these analyses have been discussed in the text. [b] These values were obtained when contact with aqueous acid solution was avoided. After exposure to *p*H 1.5 for 24–48 hours, at room temperature, benzylamine contained 1.0, and glycine ethyl ester, 0.9 equivalent of sulfate. Ethanolamine retained 1.7 equivalents under such conditions.

Free amino acids were found not to be suitable model substances for the reactivity of the amino groups in proteins, since they tended to undergo secondary reactions, leading to marked losses in amino nitrogen, without a corresponding introduction of sulfate groups. The mechanism of this reaction is not fully understood. In the case of alanine, part of the reaction product was separated by high vacuum sublimation and identified as alanine anhydride. Most of the material corresponded in its properties to a mixture of sulfamates of alanine polypeptides composed of 10–40 units.

The reactivity of the guanidyl groups of arginine was demonstrated by the considerable amount of sulfate bound by the protamine, salmine, which contains approximately 67% arginine and few other polar amino acid residues (Table III). Similarly, it was possible to introduce bound sulfate into methylguanidine (Table II). Substituted guanidine sulfuric acids have not previously been described.[8] On the other hand, imidazole groups did not appear to react readily, since acetyl-*l*-histidine bound very little sulfate (Table II).

Reaction of Thiol, Phenol, and Indole Groups.—The participation of the tyrosine and cysteine residues of proteins in the reaction was demonstrated by the disappearance of these groups as determined by the Folin methods. Sulfhydryl groups could no longer be demonstrated in a urea solution of sulfated egg albumin. Thioglycolic acid, used as a model substance, also was found to lose its thiol group upon treatment with pyridine-chlorosulfonic acid.

Tests for tyrosine with the "phenol reagent" showed that the chromogenic values of egg albumin and β-lactoglobulin were reduced by about 90% through sulfation (Table I). Hydrolysis regenerated almost all of the chromogenic groups. Thus sulfation of the phenolic hydroxyl was the predominant reaction, rather than nuclear sulfonation. This is in contrast to the action of concentrated sulfuric acid, which promoted nuclear sulfonation of the phenolic residue.[2] N-Acetyl-*l*-tyrosine and N-lauroyl-*l*-tyrosine were found to bind approximately one sulfate equivalent. The sulfated products gave no color with the phenol reagent, but most of the chromogenic value corresponding to their tyrosine contents could be regenerated by acid hydrolysis.

The ability of the indole ring to bind sulfate was indicated by the considerable amounts of sulfate that were introduced into gramicidin, a polypeptide containing about 40% of tryptophan, but few other polar groups (Table III). It was clearly demonstrated by the sulfation of indole-3-acetic acid and acetyl-*d,l*-tryptophan (Table II).

Reaction of Aliphatic Hydroxyl Groups.—That pyridine-chlorosulfonic acid reacts with hydroxyl compounds under the conditions used in this investigation has been amply shown by its use in the preparation of the sulfates of such substances as polysaccharides and polyvinyl alcohol.[5] The

(8) The analogous guanidinephosphoric acids are known. Zeile, *Z. physiol. Chem.*, **236**, 263 (1935); Deutsch and Fernö, *Nature*, **156**, 604 (1945); and others.

TABLE III

CORRELATION OF REACTIVE GROUPS[a,b] WITH SULFATE SULFUR BOUND BY VARIOUS PROTEINS AND MODEL SUBSTANCES

	Equivalents per 10^4 g. of protein						
	(1)	(2)	(3)	(4)	(5)	Sum.	Sulfate
Material	β-Hydroxy	Indole	Phenol	Total basic[b]	Amide	of cols. 1 to 5	sulfur bound[c]
Sericin	37.1	0.3	3.0	5.4	10.4	56.2	53.2
Polyglutamine	0	0	0	1.1	55	56.1	28.4
Wheat gluten	5.6	0.3	2.1	5.3	20.6	33.0	42.2
Protamine sulfate	4.3	0	0	40.0	0	44.3	23.6[d]
Gliadin	4.6	0.3	1.8	4.7	30.0	41.4	34.7
Egg albumin	9.2	0.5	2.4	8.8	7.8	28.7	29.8
Chicken feathers	13.2	0	1.9	4.5	7.4	27.0	26.8
Wool	12.3	0	3.5	8.2	10.4	34.4	26.2
Silk fibroin	13.3	0.3	5.9	1.3	2.9	23.7	23.7
Gelatin	14.2[e]	0	0.6	6.0	2.9	23.7	20.6
Globin	5.2	0.6	2.0	13.4[b]	4.5	25.7[b]	20.2
Isinglass	10.2[e]	0	0.4	9.3[f]	3.0	22.9	20.2
β-Lactoglobulin	6.3	1.0	2.4	11.6	7.1	28.4	19.3
Gramicidin	4.8	19.6	0	0	0	24.4	18.1
Polymethyl polyglutamate	0	0	0	1.4	0	1.4	2.7
Polyglutamic acid	0	0	0	1.9	0	1.9	2.2
Nylon	0	0	0	0.6	0	0.6	0.9

[a] None of the proteins listed contained more than 1 equivalent of thiol per 10^4 g. protein. This group was therefore not listed. [b] Since reliable analyses for amino and guanidyl groups are not available for all proteins, calculations were based on total basic groups determined according to ref. 12. Imidazole groups are not involved in the reaction, and should therefore be subtracted for the purpose of correlation. However, of the proteins studied only globin appears to contain more than 1.2 equivalents of histidine. Thus the error introduced is small in most cases. For globin the corrected values for columns 4 and 6 would be 8.2 and 20.5, respectively. [c] Calculated on the basis of nitrogen content of derived proteins. All analyses on air-dry basis. Sericin, gluten, and gelatin yielded soluble and insoluble fractions; the values given for these proteins are weighted averages of the sulfate contents of both fractions. Soluble products were obtained from polyglutamine, protamine sulfate, gliadin, egg albumin, β-lactoglobulin, globin, and polyglutamic acid, and insoluble products from the other proteins listed. [d] The protamine derivative could not be isolated by dialysis. The recovery of the nitrogen after precipitation of the free sulfate with barium was always low, possibly because the sulfated peptide gave an insoluble barium salt. It may thus be assumed that the fraction not recovered was the more completely sulfated. [e] Hydroxyproline content is included in these values.[3] [f] Calculated from the data of Beveridge and Lucas [*J. Biol. Chem.*, 155, 547 (1944)]. This protein did not form an insoluble dye complex.[12]

large amounts of sulfate bound by silk sericin and fibroin (Table III), both of which contain more aliphatic hydroxyl groups than other polar groups combined, is evidence that this reaction occurs also in proteins. Ethanolamine bound almost 2 moles of sulfate in comparatively stable manner, which indicated that both the hydroxyl and the amino groups had entered into the reaction (Table II).

In view of the ready sulfation of alcohols under the present conditions, the carbohydrates occurring in some proteins may be assumed to participate in the reaction. Analyses by the orcinol method[9] indicated the presence of appreciable amounts of carbohydrates in sulfated gluten and egg albumin (5 and 2%, respectively). It is noteworthy that these are the only two proteins studied which bound more sulfate sulfur than that corresponding to the sum of their reactive amino acid side chains (Table III).

Reaction of Primary Amides, and Non-reactivity of Peptide and Carboxyl Groups.—Proteins rich in amide and relatively poor in other types of polar groups, such as gliadin and gluten, bound considerable amounts of sulfate (Table III). This suggested that the primary amide groups of glutamine and asparagine reacted with pyridine-chlorosulfonic acid, a fact which was proved through sulfation of the polyamide prepared from polyglutamic acid. Thus the amide bound considerable amounts of sulfate, while the free polyglutamic acid and its polymethyl ester bound only insignificant amounts (Table III). Simple amides, such as heptamide and adipamide, were found to react readily with the sulfating agent under the conditions used with proteins (Table II). No evidence was found for the formation of disulfamates of amides, nor of any other model compound excepting the primary amines. Baumgarten and Marggraff[10] have described the preparation of N-acyl sulfamates at higher temperatures by fusion of amides with pyridine sulfotrioxide.

In contrast to the primary amides, the secondary amides of the peptide chain appeared unreactive, as indicated by the inability of various polypeptides and the polyamide, nylon, to bind appreciable amounts of sulfate (Table III). The non-reactivity of polyglutamic acid is evidence

(9) Tillmans and Philippi, *Biochem. Z.*, **215**, 36 (1929). Gluten loses most of its carbohydrate (starch) through sulfation with concentrated sulfuric acid and subsequent dialysis,[3] but only about half when sulfated under the conditions of the present paper. Control experiments showed that positive orcinol tests are given by sulfated glucose, cellulose, and starch. Through addition of starch to gluten and gliadin before sulfation, the sulfate sulfur content of the soluble fraction of the final product was increased by 0.2 and 0.6%, respectively.

(10) Baumgarten and Marggraff, *Ber.*, **64**, 1582 (1931).

that neither the carboxyl groups nor the peptide bonds play a role in determining the ability of proteins to bind sulfate groups.

Reaction in Aqueous Solution.—A few experiments were performed in aqueous solution under conditions similar to those described by Baumgarten, *et al.*[4] The sulfating agent, prepared from pyridine and chlorosulfonic acid, was isolated and washed free of pyridinium chloride by means of chloroform.[10,11] When serine was treated with this material in alkaline solution at 10°, the amino nitrogen was decreased by 46%. In the absence of sodium carbonate the reaction proceeded only to 22%. No evidence of sulfation of hydroxyl groups was found, since the sulfate sulfur contents of the isolated products were only 0.32 and 0.15 equivalent per mole, respectively. These results are in agreement with the work of Baumgarten and co-workers. When the isolated addition product was used under the usual anhydrous conditions in excess pyridine, however, it seemed to cause sulfation of the same groups of proteins and model substances, although to a somewhat lesser extent, as did the crude addition product (containing pyridinium chloride). Thus gluten sulfated by the two techniques contained 6.3 and 8.8% sulfate sulfur, respectively; indole-3-acetic acid and methylguanidine were sulfated by the isolated product to 45 and 28%, respectively. These findings illustrate the importance of the reaction conditions in determining the course of the reaction. The presence or absence of water, and the temperature, rather than the nature of the reagent, appear to be crucial in determining which groups participate in the reaction.

Stability of Sulfate Linkages.—In the preceding paper[2] the resistance of the acid sulfate esters of serine, threonine, hydroxyproline, and cysteine to acid and alkali were compared. All but hydroxyproline sulfate were found to be unstable in 1 *N* sodium hydroxide at room temperature; all appeared stable in 1 *N* hydrochloric acid.

A similar stability study has now been made of the sulfamate derivatives observed to form from amines, amides, indoles, and guanidines. Solutions of sulfated model substances were exposed to 1 *N* sodium hydroxide and 1 *N* hydrochloric acid for twenty-four hours at room temperature, and then tested for free sulfate ions. All of these classes of compounds were found to be stable in alkali. In 1 *N* acid solution only the aminomonosulfamates were stable, at *p*H 1.5 the amido sulfamates were also stable, while the guanidyl and indole sulfamates showed incipient hydrolysis at this *p*H. Acetyl-*l*-tyrosine sulfate was labile in acid but not in alkali. These observations are in agreement with published statements,[6,7] concerning the sulfated derivatives of amines, amides and phenols. It appears that sulfate derivatives of guanidines and indoles have not previously been described.

The relative stability in alkali of many of the sulfate groups introduced by means of the pyridine-chlorosulfonic acid reagent has permitted a more successful application of the dye technique[12] for the determination of total acid groups than was possible with proteins sulfated by means of sulfuric acid. These analyses showed agreement between the sulfate introduced into most proteins and the corresponding increase in acid groups (Table I), indicating that little, if any, formation of neutral sulfates or sulfamates occurred.

In the preceding study[2] of the action of concentrated sulfuric acid on proteins, the protein sulfates were found quite unstable upon prolonged standing in the reaction mixture. In contrast, sulfation with the pyridine-chlorosulfonic acid reagent for twenty-four hours instead of the usual two and one-half hours had no similarly marked effect on the extent of sulfation of several proteins and model substances.

Experimental

The protein preparations and polyglutamic acid and its derivatives were those previously used.[2] Globin hydrochloride was kindly supplied by Eli Lilly and Co. Acetyl-*d,l*-tryptophan,[13] acetyl-*l*-tyrosine,[14,15] and acetyl-*l*-histidine[16] were prepared by B. Brandon by published methods. N-Lauroyl-*l*-tyrosine was kindly furnished by E. B. Kester. The other model substances and reagents used were commercial preparations.

Sulfation Procedure.—To 183 ml. of pyridine (2.27 moles) contained in a 500-ml. wide-mouthed Erlenmeyer flask and cooled in an ice-bath, 33 ml. (0.5 mole) of chlorosulfonic acid was added dropwise with mechanical stirring. The chlorosulfonic acid, contained in a dropping funnel, was protected from moist air by a calcium chloride drying tube. Dry natural gas was blown through the reaction vessel during the addition of the acid in order to remove as much hydrogen chloride as possible.

The usual procedure was as follows: For each gram of material to be sulfated, 20 ml. of the freshly prepared sulfating mixture[17] was introduced into an Erlenmeyer flask. To prevent lumping, it was necessary to add the proteins in small portions with stirring. The flask was closed with a cork carrying a stirring rod, and the mixture was heated for two and one-half hours in an air oven at 75–78°. The flasks were removed at intervals and the contents stirred. At the end of the reaction period the mixtures were poured into water. The resultant solution was at approximately *p*H 5. In cases where it appeared important to avoid contact with aqueous acid, the reaction mixtures were poured into water maintained above *p*H 8 by simultaneous addition of alkali. In some cases the reaction mixture was poured into a stirred suspension of barium carbonate and barium hydroxide. The purpose was threefold: to remove inorganic sulfate, maintain an alkaline medium, and avoid the formation of large amounts of soluble salts.

For most experiments with proteins, the diluted reaction mixtures were brought to neutrality with 1 *N* sodium

(11) This product contained 18.7% sulfate sulfur, that obtained from pyridine and sulfur trioxide 18.3%. Calculated for $C_5H_5NSO_3$: 20.1%. Both products melted at 97–100°, considerably lower than the highest melting point (175°) given by Baumgarten (*Ber.*, **59**, 1166 (1926)).

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

(13) Berg, *ibid.*, **85**, 207 (1929–1930).

(14) du Vigneaud and Meyer, *ibid.*, **98**, 295 (1932).

(15) Niemann and McCasland, THIS JOURNAL, **66**, 1870 (1944).

(16) Bergmann and Zervas, *Biochem. Z.*, **203**, 284 (1928).

(17) When stored at −18°, the sulfating mixture was fully effective after eight days, as measured by the amount of sulfate introduced into gliadin.

hydroxide and dialyzed until free of inorganic sulfates. Any insoluble material was separated and dried with organic solvents. The soluble portion was concentrated to small volume in dialysis tubing hung in a current of warm air, and then dried from the frozen state. The air-dried samples were analyzed for nitrogen and sulfate sulfur by a modification of the Mease method.[2,18] For substances of low molecular weight, the inorganic sulfates were removed as barium sulfate, and an aliquot of the resultant solution was analyzed for sulfate sulfur.[2,18] Another aliquot was freed from pyridine by repeated evaporation to dryness at room temperature from alkaline solution; it was then analyzed for nitrogen by the Kjeldahl procedure. Control experiments showed that a small fraction (about 0.4%) of the nitrogen of the crude pyridine-chlorosulfonic mixture became non-volatile when the material was heated to 75–78° for two and one-half hours, and progressively more with longer heating. This nitrogenous residue contained some bound sulfate, the S/N ratio being 0.5 to 0.7. Less of this material was formed if the sulfating agent had been isolated and resuspended in pyridine before being heated. Thus an inconstant error, apparently depending upon the nature of the substance being sulfated, detracts from the quantitative significance of the data obtained with small-molecular model substances, as listed in Table II.

In analyzing for sulfate sulfur, the usual conditions of hydrolysis (one hour at 100° in 6 *N* HCl) were found to be not quite sufficient for the quantitative release of the sulfate from certain proteins. A possible explanation is that hydrolysis of sulfated amide groups occurs in such a manner that sulfamic acid is primarily released rather than sulfuric acid.[10] Sulfamic acid is known to be relatively resistant to hydrolysis,[7] and was found to yield only 90% of its sulfate sulfur under our conditions of hydrolysis. When hydrolyzed by refluxing at 120° for sixteen hours, conditions which led to complete breakdown of sulfamic acid, some proteins, notably polyglutamine and β-lactoglobulin, showed sulfate sulfur contents higher by as much as 12%, while others did not. The values in Table III, however, were obtained after one hour of hydrolysis.

All other analytical methods were the same as those used in the preceding study.[2]

Products of Reaction of Pyridine-chlorosulfonic Acid with Alanine.—Five grams of alanine was treated with the pyridine-chlorosulfonic acid reaction product in the usual manner. The mixture was then diluted with water, neutralized, freed from sulfate ions and evaporated to dryness while maintaining an alkaline reaction. The residue was extracted repeatedly with absolute alcohol; 280 mg. of nitrogen dissolved, an equal amount remained in the insoluble fraction. The residue obtained upon evaporation of the alcohol contained 13.8% N, 3.04% sulfate S, 3.50% total S and 0.25% amino N. After acid hydrolysis the amino nitrogen agreed with the total nitrogen. The S/N ratio of 0.12, together with the amino N/N ratio of 0.02 suggests that free as well as terminally sulfated polypeptides must be present. The occurrence of alanine anhydride in this mixture was demonstrated by separating it by high-vacuum sublimation from the crude product; 320 mg. was obtained from 1 g. of product.

Anal. Calcd. for $C_6H_{10}N_2O_2$: N, 19.7. Found: N, 19.8; amino-N, 0.1.

Acknowledgment.—The authors are indebted to S. Ahnger and C. Cleaver for numerous analytical determinations.

Summary

Various proteins were treated with the reaction product of chlorosulfonic acid and pyridine under anhydrous conditions at 70–80° for two and one-half hours. This led to the covalent binding of considerable amounts of sulfate sulfur (up to 10%), corresponding in several proteins to the sulfation of one out of every three amino acid residues. The mode of linkage was ascertained through the use of model substances and proteins or polypeptides rich in certain groups. It was thus shown that, under the condition used, part or all of the aliphatic and phenolic hydroxyl, thiol, primary amide, amino, guanidyl and indole groups were transformed to sulfates or sulfamates. For most proteins, there was a definite correlation between the sum of these groups and the sulfate introduced. The imidazole and carboxyl groups and the peptide linkage did not participate in the reaction.

The stability of the various sulfate linkages in acid and alkali was investigated. Sulfamates of indoles or guanidines appear not to have been described previously.

(18) Mease, *J. Res. Nat. Bur. Stand.*, **13**, 617 (1934).

ALBANY, CALIF. RECEIVED APRIL 8, 1946

[CONTRIBUTION FROM THE MERCK RESEARCH LABORATORIES]

Substituted Sulfaquinoxalines. I. The Isolation and Synthesis of 3-Hydroxy-2-sulfanilamidoquinoxaline and of Related Quinoxalines

BY J. R. STEVENS,[1] K. PFISTER, 3RD, AND F. J. WOLF

The drug 2-sulfanilamidoquinoxaline[2] (I) is of particular interest as a prophylactic agent in avian malaria and exhibits certain unique characteristics not generally found in the sulfa drugs.[3] In experiments on its chronic toxicity in rats, the formation of calculi in the tubule region of the kidneys was observed,[3] and the importance of the structure of this product in considering the metabolic fate of the drug became important.[4]

Examination of the crude calculi indicated that the compound present was not unchanged sulfaquinoxaline although a positive Marshall test established the presence of a free aromatic amino group. Analyses of the purified material proved that it was a hydroxylated derivative of sulfa-

(1) Present address: J. T. Baker Chemical Company, Phillipsburg, New Jersey.

(2) J. Weijlard, M. Tishler and A. E. Erickson, THIS JOURNAL, **66**, 1957 (1944).

(3) A. O. Seeler, C. W. Mushett, C. Graessle and R. Silber, *J. Pharmacol.*, **82**, 357 (1944).

(4) Scudi and Silber, *J. Biol. Chem.*, **156**, 343 (1944), isolated 3-hydroxy-2-sulfanilamidoquinoxaline from the urine of rabbits receiving 2-sulfanilamidoquinoxaline. The metabolic product was identified by degradation with hydrochloric acid to 2,3-dihydroxyquinoxaline.