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THE MECHANISM OF SULFUR LABILITY IN CYSTEINE AND ITS DERIVATIVES. I. SOME THIO ETHERS READILY SPLIT BY ALKALI

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It is the purpose of the present paper, first, to describe the behavior of certain ketonic derivatives of thio ethers which are somewhat remarkably labile toward alkali, and, afterward, to point out a probable relation between the behavior of these compounds and the problem of "labile sulfur" in materials of biological interest.

Generally speaking, the thio ethers, RSR′, are very stable toward all hydrolytic agents. Even the mercaptans of hydrocarbon radicals are quite stable toward acids and alkalies. Recently Billheimer and Reid[1] have shown that certain aliphatic mercaptans, when heated for two hours at 260° with 3 *N* alkali, are only partially hydrolyzed, and these results may perhaps be taken as typical. Vorländer[2] found, not unexpectedly, that triphenylmethyl mercaptan was rather readily hydrolyzed.

Really striking lability of the single bond[3] between carbon and divalent sulfur, however, is to be found only in derivatives containing other substituents, suitably located, and of these the most effective appears to be the keto group.

The α-keto derivatives, $RCOCH_2SR'$, show in some cases considerable instability of the sulfur–carbon bond, particularly toward alkaline and reducing reagents, and it is of interest that no compound of the type $RCOCH_2SH$ appears to have been isolated in a state of purity. The hydrolytic splitting of such compounds seems, however, to have little relation to the common reactions of biological sulfur compounds, as it is frequently, if not generally, attended by the oxidation of the sulfur[4] and its replacement by hydrogen.

Particular attention must be paid, however, to the β-keto sulfides, $RCH(SR')CH_2COR''$. A number of these compounds have been made, chiefly by Ruhemann[5] (see also Posner[6]). The reaction involved is the

[1] Billheimer and Reid, THIS JOURNAL, **52**, 4338 (1930).

[2] Vorländer and Mittag, *Ber.*, **46**, 3450 (1913).

[3] The thio acids and their esters, as well as thiourea derivatives, are substances, the hydrolysis of which is well known and fairly well understood. They are expressly omitted from the present discussion.

[4] The compound $C_6H_5COCH(SCH_2COOH)C_6H_5$ (desylthioglycolic acid), for instance, deposits desoxybenzoin when dissolved in cold dilute alkali. This reaction is being studied and the results will be reported later.

[5] Ruhemann, *J. Chem. Soc.*, **87**, 461 (1905).

[6] Posner, *Ber.*, **35**, 809 (1902).

addition of a mercaptan to an α,β-unsaturated ketone, preferably with the aid of an alkaline catalyst, as in Equation A and is doubtless to be considered as a 1,4-addition to the conjugated series of double bonds.

$$RCH{=}CHC(=O)R'' + R'SH \rightleftharpoons RCH(SR')CH{=}CR''(OH) \rightleftharpoons RCH(SR')CH_2C(=O)R'' \quad (A)$$

Ruhemann and Posner were both primarily interested in the sulfones to be obtained by the oxidation of their products, and neither appears to have noticed that, in the presence of alkali, the reaction represented in Equation A is quite easily reversed, and the initial products regenerated.[7]

Reading from right to left, Equation A represents this reversal as a 1,4-elimination from the enol form, with regeneration of the original conjugated unsaturated system. Easy reaction would accordingly be expected also for a substance of the type $RCH(SR')CH{=}CHCH_2COR''$, from the enol form of which 1,6-elimination of H and SR′ should take place. But this latter case seems to have no present biochemical significance.

In the Experimental Part the preparation of certain of these β-keto sulfides and their behavior when treated with alkalies are described. Two examples may be discussed briefly at this point.

From benzalacetophenone and thioglycolic acid, the sulfide acid $C_6H_5CH(SCH_2COOH)CH_2COC_6H_5$(I) was prepared. It does not dissolve in dilute caustic alkalies to give a clear solution, because of the ease with which benzalacetophenone is liberated. In 0.1 *N* sodium carbonate solution at room temperature it is more than one-third decomposed in one hour, as measured by the weight of crystalline benzalacetophenone of correct melting point obtained when the solution is filtered. The reaction goes to completion on longer standing. The ease of splitting is here very strikingly greater than that of the "labile sulfur" of, for instance, insulin, as determined by Abel and Geiling.[8]

In the case of β-*p*-tolylmercapto-benzylacetophenone, $C_6H_5CH(SC_6H_4CH_3)CH_2COC_6H_5$ (II), and similar compounds, the lack of a carboxyl group makes experiments in water difficult, as the substances dissolve appreciably neither in water nor in alkali. When, however, the substance II is dissolved in alcohol together with an excess of lead acetate (no reaction occurs at this stage) and treated with sufficient sodium hydroxide solution to make the final alkali concentration 0.1 molar, the yellow lead salt of *p*-tolylmercaptan is rapidly precipitated at room temperature. If the reaction is stopped after one minute by the addition of an excess of acetic acid, the decomposition is found to have been nearly quantitative,

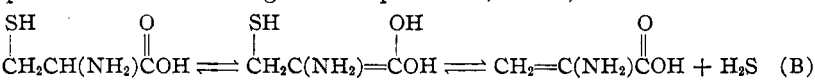
[7] Kohler and Reimer [*Am. Chem. J.*, **31**, 163 (1904)] have, however, made brief mention of the fact that the analogous sulfones are readily split by alkali to give sulfinates and unsaturated ketones.

[8] Abel and Geiling, *J. Pharmacol.*, **25**, 423 (1925).

as measured by the weight of the pure lead salt obtained. Other analogous derivatives were correspondingly reactive. Moreover, the substance II, when warmed on a steam-bath with a slight excess of phenylhydrazine in acetic acid, readily gives the sulfur-free 1,3,5-triphenylpyrazoline.

It should, perhaps, be emphasized here that the combined work of Ruhemann,[5] Kohler[7] and Posner[6] leaves no reasonable doubt as to the structures of thio ether derivatives of the type described.

Suggested Mechanism of the Reaction of Cysteine with Alkali.— In the previous section the action of alkalies on certain β-keto thio ethers has been discussed, but inspection of the formula of cysteine shows that it also has its —SH group attached in the β-position with reference to a —CO— group, and although the latter forms part of a carboxyl group, there is at least a formal analogy between the two types of compounds. Equation B is thus analogous to Equation A, above, reversed.



Equation B thus represents the idea that the removal of sulfur from cysteine by alkalies is due primarily to the presence of the sulfur in the position β- to the carboxyl group, and suggests that it takes place as a 1,4-elimination of hydrogen sulfide[9] from an intermediate enolized form. Various reasons for such a view will now be given.

That alkaline decomposition of cysteine (or cystine) involves the removal of H_2S (or H_2S_2) from the molecule, rather than simple hydrolysis (replacement of —SH by —OH), seems fairly certain and, incidentally, constitutes a striking analogy to the reaction of the β-keto sulfides. Clarke and Inouye[10] have recently given additional reasons for believing that aminoacrylic acid is one of the early products of the reaction. Baumann[11] long ago stated that S-phenylcysteine loses phenylmercaptan when heated with alkali, at least as rapidly as cysteine loses hydrogen sulfide under similar conditions.

It is, on the other hand, a well-known fact that the tendency to enolization of a carboxyl group is likely to be very small indeed compared to that of a keto group. Is the tendency in this particular case sufficient to justify the theory presented? Fortunately, evidence is available to test this point. There is very little doubt that the racemization of an optically active α-amino acid by alkali, when it occurs, is due to just this enolization process. Most of the common amino acids of biochemical interest show very little tendency to racemize except under quite extreme conditions. But serine (Daft and Coghill[12]) is apparently very readily

[9] From cystine, RSSH, or eventually H_2S_2, would be split out.

[10] Clarke and Inouye, *J. Biol. Chem.*, **89**, 399 (1930).

[11] Baumann, *Ber.*, **15**, 1733 (1882).

[12] Daft and Coghill, *J. Biol. Chem.*, **90**, 341 (1931).

racemized by surprisingly dilute alkali, and the careful measurements of Andrews[13] on its analog, cystine, show that the latter is slowly racemized even by cold alkali, and at a rate several times (but not many times) more rapid than the rate of sulfur loss under the same conditions. This is as it should be, for enolization must cause racemization, but need not necessarily lead, in any given instance, to the loss of sulfur.

The loss of sulfur in alkaline cystine solutions is a relatively slow reaction. With boiling normal alkali, in the presence of excess lead oxide, it is about half complete in an hour (Clarke and Inouye[10]). The decomposition of the β-keto sulfides is so enormously more rapid than this that there seems to be no difficulty on this ground in accepting the suggested mechanism, as represented by Equation B.

But certain derivatives of cysteine and of cystine are known in which the sulfur is very much more labile than in the amino acids themselves. Referring only to substances of known constitution, glutathione shows very strikingly greater sulfur lability than does cysteine (Hopkins[14]). Dialanylcystine loses hydrogen sulfide (and sulfur) somewhat more readily than cystine, but very much less readily than does its anhydride.[15,16] Derivatives of cystinehydantoin[17,18] also show conspicuous reactivity. These results are of interest in relation with the fact that many proteins (and, of course, insulin) contain a portion of their sulfur in a form conspicuously more labile than that of cystine itself.

In this connection, attention must be called to the fact that modifications of the carboxyl group such as ester or amide formation are well known to increase the tendency toward enol formation with the hydrogen of the α-carbon atom. Applications of this fact in the biochemical field may be seen in the work of Dakin[19] and Levene[20] on the racemization of peptides and diketopiperazine derivatives. A detailed discussion of this point does not seem to be called for here, but it has already been pointed out that increased ease of racemization (of a cysteine or cystine residue) and increased ease of desulfurization should be expected to go together.

The conclusion to be drawn is that, on the basis of the theory presented in this paper, it would be expected that certain cystine derivatives should be more sensitive to desulfurization by alkali than cystine itself; and this expectation of increased sensitivity would apply specifically to those

[13] Andrews, *J. Biol. Chem.*, **80**, 191 (1928).

[14] Hopkins, *ibid.*, **84**, 276 (1929).

[15] Bergmann and Stather, *Z. physiol. Chem.*, **152**, 193, 198 (1925).

[16] Brand and Sandberg, *J. Biol. Chem.*, **70**, 381 (1926).

[17] Bergmann and Delis, *Ann.*, **458**, 76 (1927).

[18] Nicolet, *J. Biol. Chem.*, **88**, 403 (1930).

[19] Dakin, *ibid.*, **13**, 357 (1912); Dakin and Dudley, *ibid.*, **15**, 263 (1913).

[20] Levene and Pfaltz, *ibid.*, **63**, 661 (1925); **68**, 277 (1926).

derivatives in which the carboxyl group of cystine is in peptide or analogous combination. Cystine peptides in which the carboxyl group remains free should, on the whole, be distinctly less reactive, not necessarily than cystine itself, but than somewhat similar peptides in the formation of which the cystine carboxyl takes part.[21] As glutathione (the reduced form) is known[22] to be γ-glutamyl-cysteyl-glycine, its special sensitivity is in accord with this expectation.

The existence of decidedly "labile" sulfur in many biological products has suggested to a number of workers in the past that these products must contain a portion of their sulfur in some unknown form, certainly different from cysteine. It should be pointed out that, as cysteine derivatives are known which show greatly increased sulfur lability, and as their behavior is only what would be expected on the basis of the ideas here presented, "labile sulfur" is scarcely to be considered as an indication of the presence of novel types of sulfur compounds.

Experimental

Benzylacetophenone-β-thioglycolic Acid, $C_6H_5CH(SCH_2COOH)CH_2COC_6H_5$, (I).—Thioglycolic acid (2.0 cc.) was dissolved in 25 cc. of absolute alcohol, and 2 *N* sodium ethylate (in absolute alcohol) added until 0.5 cc. more was present than necessary for neutralization to phenolphthalein. One mole of benzalacetophenone (5.2 g.) was then added, and the solution allowed to stand overnight. It was then just neutralized with acetic acid, poured into 100 cc. of 3% sodium carbonate solution and filtered promptly with suction from a gelatinous precipitate into a flask containing an excess of acetic acid. A crystalline precipitate formed, which melted at 120° and after two recrystallizations from five parts of alcohol melted constantly at 129°. The yield was about 40%.

Anal. Calcd. for $C_{17}H_{16}O_3S$: S, 10.67. Found: S, 10.72, 11.12 (Parr bomb).

If more alkali is used in the condensation, the amount of the precipitate is larger, and the acid cannot readily be obtained crystalline. The gelatinous precipitate dissolved with some difficulty in hot alcohol and after five recrystallizations melted at 199–200°. It was tentatively identified as the low-melting form of 1,3,5-tribenzoyl-2,4-diphenylpentane.

Hydrolysis of the Acid, I.—Half a gram of the pure acid was added to 25 cc. of normal sodium hydroxide. The solution was turbid from the beginning and soon deposited a heavy precipitate of crystalline material. On filtration after fifteen minutes a good yield of benzalacetophenone, melting at 55.5°, was obtained.

Even in 0.1 *N* sodium carbonate, a really clear solution of the acid could not be obtained. On standing at room temperature, the turbidity of the solution gradually increased, and after one and one-half hours filtration separated more than half of the calculated amount of benzalacetophenone. On longer standing, a further quantity separated. The solution contained thioglycolic acid. It is thus apparent that even under

[21] It is almost obvious that the adjacent amino group would also exert an influence on the firmness of the sulfur bond. Modification of the amino group would therefore have its effect. It is, however, believed that the effect of the carboxyl, and particularly of the carboxamido group, is of decidedly greater importance.

[22] Nicolet, *J. Biol. Chem.*, **88**, 389 (1930); Kendall, Mason and McKenzie, *ibid.*, **88**, 409 (1930); Grassmann, Dyckerhoff and Eibler, *Z. physiol. Chem.*, **189**, 112 (1930).

these very mild conditions the material breaks down to give the substances from which it was formed. It was to prevent, in so far as possible, a similar decomposition, that the crude solution of the sodium salt, in the preparation of the acid, was filtered directly into acetic acid.

Benzylacetone-β-thioglycolic Acid, $C_6H_5CH(SCH_2COOH)CH_2COCH_3$.—This acid was prepared essentially as described above, but from benzalacetone. It has not yet been obtained in crystalline condition, and its detailed description is therefore postponed.

Benzylacetophenone - β - *p* - tolylsulfide, $C_6H_5CH(SC_6H_4CH_3)CH_2COC_6H_5$ **(II)**[5].—Molar proportions of benzalacetophenone (8.4 g.) and *p*-tolylmercaptan (5.0 g.) were dissolved in 40 cc. of benzene, and a little 2 *N* sodium ethylate solution added (0.5 cc. more than required to give a pink color with phenolphthalein). After standing overnight, 2 cc. of acetic acid and 25 cc. of alcohol were added. The product (crude yield 86%) was best purified by solution in its own weight of warm chloroform and addition of warm alcohol. It is soluble in hot alcohol to the extent of 7–8%, but quite difficultly soluble in cold alcohol. It melts at 113°.

Anal. Calcd. for $C_{22}H_{20}OS$: S, 9.64. Found: S, 9.72, 9.72 (Parr bomb).

When this sulfide was dissolved in acetic acid and warmed on the steam-bath with a slight excess of phenylhydrazine, *p*-tolylmercaptan was split out and 1,3,5-triphenylpyrazoline was easily formed in good yield. The product was identical with that formed from benzalacetophenone and phenylhydrazine.

Benzylacetone - β - *p* - tolylsulfide, $C_6H_5CH(SC_6H_4CH_3)CH_2COCH_3$ **(III).**—The preparation from benzalacetone followed closely that for its analog, II. The reaction was stopped by the addition of acetic acid without alcohol, the benzene solution evaporated, and the quite soluble residue crystallized from a small volume of alcohol. The pure substance melts at 64°.

Anal. Calcd. for $C_{17}H_{18}OS$: S, 11.85. Found: S, 11.96, 11.81 (Parr bomb)

Alkaline Hydrolysis of the Sulfides II and III.—Due to the insolubility of these substances in water and in alkali, it was necessary to use alcoholic solutions, but since the reaction with alkali is reversible, an idea of its rate can only be got when one of the products is removed as it is formed. Accordingly, the following procedure was used.

One gram of the sulfide II was dissolved in 86 cc. of warm alcohol, and the solution rapidly cooled to 30–35° (it is then supersaturated). Twice the calculated amount of 20% lead acetate solution was then added (no coloration or precipitate was produced) followed by sufficient 6 *N* alkali to neutralize the acetic acid from the acetate and leave the solution 0.5 *N* with regard to alkali. A yellow precipitate began to form at once and after ten minutes an excess of acetic acid was added to stop the reaction. The orange precipitate soon became yellow and crystalline. It weighed 0.6 g. and was practically pure $Pb(SC_6H_4CH_3)_2$, melting at 205–208° to an orange-red oil. On treatment of the lead salt with potassium iodide and iodine, di-*p*-tolyl disulfide was obtained in good yield (m. p. 45.5°) and fully identified.

When alkali concentrations as low as 0.1 *N* were used, an equally good yield of the lead mercaptide was obtained, and this was still true when the time of action of the alkali was reduced to one minute. Control experiments showed that reaction occurred only while the solution was alkaline.

The behavior of the sulfide III was similar in all respects.

Summary

Certain β-ketonic sulfides have been described which are split by alkali with rather extreme ease to give mercaptans and α,β-unsaturated ketones.

A structural analogy between these compounds and cysteine or cystine has been pointed out, and some of the reasons have been given for considering the action of alkali on the two classes of compounds as analogous. From these considerations, a mechanism for the alkaline decomposition of cysteine has been developed and reasons have been indicated for the anticipation of greatly increased reactivity in certain specified types of cysteine and cystine derivatives.

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ROTENONE. XIII. OXIDATION OF METHYLDERRITOLIC ACID AND THE SYNTHESIS OF 2,3,5- AND 2,3,6-TRIMETHOXYBENZOIC ACIDS AND THEIR DERIVATIVES

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As reported in previous articles,[1] derritol methyl ether is converted by catalytic hydrogenation into methylderritolic acid, which is oxidized by hydrogen peroxide in alkaline solution to a compound of the formula of a trimethoxyphenylacetic acid. We have also reported that this trimethoxyphenylacetic acid is oxidized by permanganate to a trimethoxybenzoic acid melting at 78–80.°

Four of the six trimethoxybenzoic acids have been definitely described in the literature, but since the properties of none of the known acids agree with those of the oxidation product of the trimethoxyphenylacetic acid from derritol methyl ether, it seemed necessary to prepare the two missing members, that is, the 2,3,5- and 2,3,6-trimethoxybenzoic acids.

Orthovanillin was used as the starting material, the methylation of which was carried out according to the method of Späth and Mosettig.[2] The methyl ether was nitrated by the method of Perkin and Robinson[3] and the isomeric 2,3-dimethoxy-5-nitro and 2,3-dimethoxy-6-nitrobenzaldehydes were separated by means of their *p*-toluidides.[4]

The first-mentioned compound was converted into 2,3-dimethoxy-5-hydroxybenzaldehyde, which was then methylated and oxidized to the 2,3,5-trimethoxybenzoic acid, which melted at 105°.

The 2,3,5-trimethoxyphenylacetic acid was prepared from the corresponding aldehyde by the Mauthner synthesis.

The 2,3,6-trimethoxybenzoic acid was prepared by first oxidizing the 2,3-dimethoxy-6-nitrobenzaldehyde and then replacing the nitro group

[1] LaForge and Smith, THIS JOURNAL, **52**, 1088, 4597 (1930).

[2] Späth and Mosettig, *Ann.*, **433**, 144 (1923).

[3] Perkin and Robinson, *J. Chem. Soc.*, **105**, 2389 (1914).

[4] Perkin, Robinson and Stoyle, *ibid.*, **125**, 235 (1924).