[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, POLYTECHNIC INSTITUTE OF BROOKLYN]

The Relative Ease of Oxidation and Alkylation Reactivity of Monomeric and Polymeric Sulfhydryl Groups[1]

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A kinetic study was made to determine the ease of oxidation and the alkylation reactivity of polymers containing sulfhydryl groups and their low molecular weight analogs of similarly constituted environment. The relative rates of oxidation were compared on the basis of the spectrophotometrically observed rate of reduction of a dye, sodium 2,6-dichlorobenzeneoneindophenol, occurring on reaction with these thiol groups. The alkylation reactivity of these thiols was compared by following the decrease in sulfhydryl concentration occurring on reaction with ω-bromoundecanoic acid in aqueous *p*H 10 buffer. *p*-Thiocresol, 2,4-di-(*p*-mercaptophenyl)-pentane and thioglycolic acid were examined as monomeric sulfhydryl groups and the polymeric sulfhydryl groups were those which resulted from the hydrolysis of a homopolymer of *p*-vinylphenyl thioacetate and a copolymer of *p*-vinylphenyl thioacetate and methyl methacrylate. A suggestion based on intromolecular disulfide bond formation is offered for the relatively fast rate of oxidation observed for the sulfhydryl groups of the hydrolyzed homopolymer and 2,4-di-(*p*-mercaptophenyl)-pentane. For the reaction with ω-bromoundecanoic acid, second-order kinetics were observed in the cases of the monomeric thiol compounds. Second-order kinetics were not obeyed by the polymeric sulfhydryl compounds; the reaction rate decreased rapidly with time, most probably because of steric hindrance to continued alkylation of adjacent sulfhydryl groups.

The activity of many enzymes has been shown to depend on the presence of sulfhydryl groups,[3] not all of which are of equal reactivity nor of equal importance for the proper functioning of the enzyme. Three types of sulfhydryl groups are recognized depending on the reactivity with sulfhydryl reagents. These have been classified loosely as freely reacting, sluggishly reacting and "masked."[4] The "masked" groups become freely reactive when the protein is denatured. This is believed due to the unfolding of the peptide chains, thus making these hitherto inaccessible sulfhydryl groups freely available.[5] Resistance to oxidation of some of the sulfhydryl groups of the enzymes is thought to be due to the distance between sulfhydryl groups in the native protein, which prevents the formation of a disulfide bond. Barron has shown that for a series of dithiols, the greater the distance between the thiol groups the slower will be the oxidation.[6] The presence of electronegative groups in a molecule also was found to influence the ease of oxidation of a series of dithiols,[6] the effect being a decrease in the degree of dissociation of the SH bond resulting in a decrease in the rate of oxidation. The influence of *p*H on the rate of oxidation of thiol groups is also in the same direction, oxidation being most rapid at higher *p*H's where dissociation of the SH bond is more nearly complete.[7]

Synthetic sulfhydryl polymers recently have been prepared by the hydrolysis of the polymer of *p*-vinylphenyl thioacetate and also the copolymer of *p*-vinylphenyl thioacetate with methyl methacrylate.[8] The ability of the hydrolyzed copolymer to reactivate urease, a natural sulfhydryl enzyme, which had been inactivated by oxidation with iodine suggested its possible use as a radiation prophylactic.[9] In particular, we had hoped that the polymer might be immobilized and not eliminated by cross-linking through disulfide linkages allowing some isolation of free sulfhydryl groups for more permanent protection. The damaging effect of X-ray radiation is believed due to the oxidizing action of $\cdot O_2H$ and $\cdot OH$ radicals and hydrogen peroxide known to be produced by irradiation of water containing dissolved oxygen.[10] Sulfhydryl compounds such as cysteine and glutathione in relatively large amounts counteract this effect by detoxifying these radicals, most probably by a rapid reaction of these radicals with the thiol groups, a reaction of relatively low activation energy. Thus the efficiency of a sulfhydryl radiation prophylactic has been shown to be related to its ease of oxidation.[11]

It was of interest, therefore, to determine the rate of oxidation and alkylation reactivity of the sulfhydryl groups of these hydrolyzed polymers of *p*-vinylphenyl thioacetate which may be considered as models for proteolytic sulfhydryl enzymes. Specifically the extent to which the oxidation of the sulfhydryl group is affected by the restraints of a polymeric chain in preventing the formation of a disulfide bond was one purpose of this investigation. For comparison purposes, 2,4-di-(*p*-mercaptophenyl)-pentane was prepared[1] as a model compound representing the repeating segment of the hydrolyzed homopolymer. Other monomeric sulfhydryl compounds which were compared were *p*-thiocresol and thioglycolic acid. Alkylation reac-

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[1] This is the 17th in a series of papers on new monomers and polymers. For the previous paper in this series see C. G. Overberger and P. V. Bonsignore, THIS JOURNAL, **80**, 5427 (1958).

[2] This paper comprises a portion of a dissertation submitted by P. V. Bonsignore in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the Polytechnic Institute of Brooklyn.

[3] (a) J. B. Sumner and K. Myrback "The Enzymes," Vol. I, Academic Press, Inc., New York, N. Y., 1951, pp. 325 *et seq.*; (b) E. S. G. Barron, "Advances in Enzymology," Vol. 11, Interscience Publishers, Inc., New York, 1951, p. 232 *et seq.*

[4] Ref. 3b, p. 219.

[5] A. E. Mirsky and L. Pauling, *Proc. Natl. Acad. Sci. U. S.*, **22**, 439 (1936).

[6] E. S. G. Barron, Z. B. Miller and G. Kalnitsky, *Biochem. J.*, **41**, 62 (1947).

[7] E. S. G. Barron and C. M. Lyman, *J. Biol. Chem.*, **121**, 275 (1937).

[8] C. G. Overberger and A. Lebovits, THIS JOURNAL, **78**, 4792 (1956).

[9] We are indebted to Professor H. Morawetz for the initial suggestion to test these sulfhydryl polymers as radiation prophylactics. Results to be reported elsewhere have demonstrated that the hydrolyzed copolymer of *p*-vinylphenyl thioacetate and methyl methacrylate is about as successful as a radiation prophylactic as any small molecule. However, as with all compounds tested to date, they are not effective if given to mice more than 15 minutes before a lethal dose of irradiation.

[10] A. Hollaender, "Radiation Biology," Vol. I, McGraw-Hill Book Co., Inc., New York, N. Y., 1954, pp. 256, 274, 284 and 1008.

[11] D. G. Doherty, W. T. Burnett, Jr., and R. Shapiro, *Radiation Research*, **7**, 13 (1957).

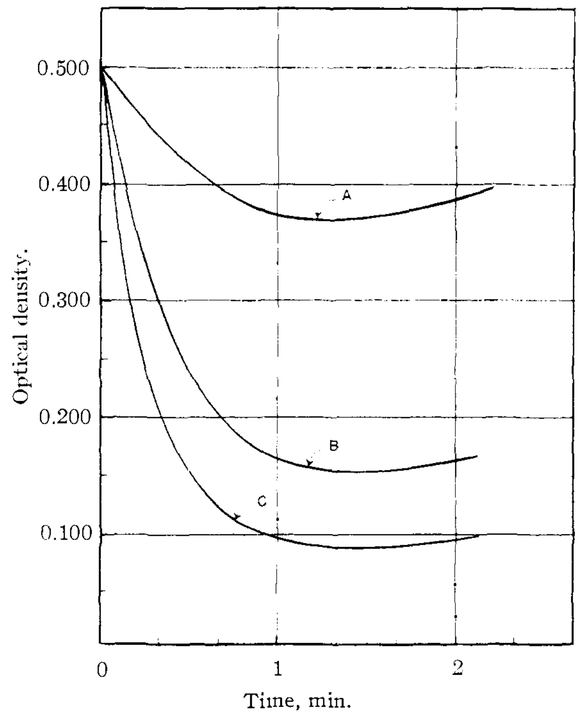

Fig. 1.—Rate of disappearance of 605 mμ peak of 2,6-dichlorobenzeneoneindophenol on reaction with the hydrolyzed homopolymer at various ratios of thiol to dye: A, 1.84; B, 3.67; C, 5.50.

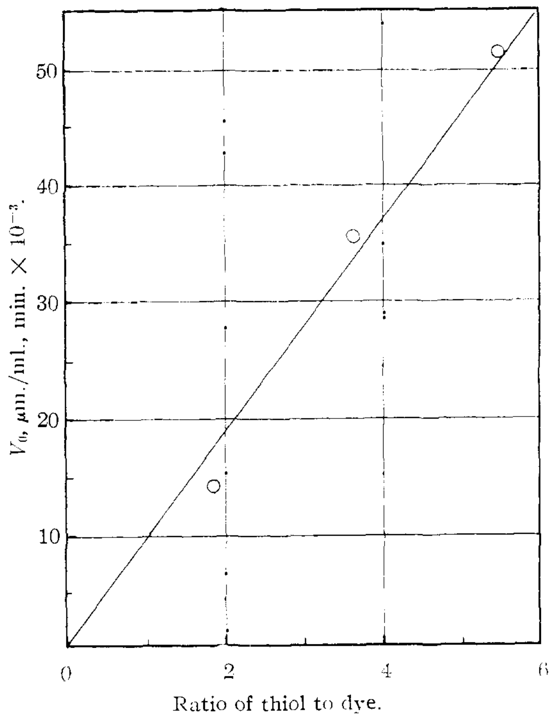

Fig. 2.—Relation between initial reaction velocity of reduction V_0 of indophenol to the ratio of thiol to dye for the hydrolyzed polymer.

tivities also were examined to observe whether the decreased mobility and diffusibility of a mercaptide anion attached to a polymeric chain significantly diminished its nucleophilicity.

Experimental

Oxidation Reactivity.—The method was the one devised by Basford and Huennekens[12] based on the spectrophotometric reduction of a dye, sodium 2,6-dichlorobenzeneoneindophenol. A sample of the dye (Eastman Kodak Co.) purified to $99^{+}\%$ purity by chromatography[13] was determined to have a molar extinction coefficient at 605 mμ of 1.82×10^4 at *p*H 10. Basford and Huennekens gave an ϵ value at 600 mμ of 1.91×10^4 at *p*H 7.0. The rates of oxidation for all the thiols investigated were carried out at a buffered *p*H of 10 since the hydrolyzed homopolymer of *p*-vinylphenyl thioacetate was insoluble below *p*H 10. The water used to prepare the buffer was boiled for 15 minutes and cooled under nitrogen in order to remove traces of oxygen since at the dilute solution concentration of thiols used and the relatively high *p*H of 10, auto-oxidation of thiols is very facile.[5] The hydrolyzed homopolymer of *p*-vinylphenyl thioacetate and the copolymer of *p*-vinylphenyl thioacetate and methyl methacrylate were prepared according to the procedure of Overberger and Lebovits.[6] The concentrations of sulfhydryl groups in solutions of these hydrolyzed polymers were determined by the *p*-mercurichlorobenzoic acid method of Boyer,[14] a method which in control experiments was found to be accurate within 1%. All the spectrophotometric work was done with a Beckman model DU spectrophotometer.

Procedure.—In general, 1 ml. of the dye solution, whose concentration was known from the optical density at 605 mμ, was placed in the spectrophotometric cuvette, and 2 ml. of the appropriate thiol solution, both buffered at *p*H 10, was injected from a hypodermic syringe as rapidly as possible into the cuvette. The optical density at 605 mμ was followed at approximately 20-second intervals. The amount of thiol oxidized was assumed to be equal to the amount of dye reduced and was obtained by subtracting the optical density at 605 mμ at any time from the initial value of the optical density and dividing by the molar extinction coefficient. The blank was the *p*H 10 borate buffer. The initial velocity, V_0, was determined from the value of the optical density at 605 mμ at 20 seconds and was expressed as micromoles of indophenol reduced per ml. per minute. The reduced initial velocity V_0' by converting to a ratio of thiol to dye of 1.1 was used for comparison purposes. The validity of this procedure is illustrated in Figs. 1 and 2 which show the proportionality which exists between thiol to dye ratio and V_0.

Alkylation Reactivity.—ω-Bromoundecanoic acid was prepared by the method of Jones.[15] Its ultraviolet absorption at *p*H 10 in the range 250–280 mμ was found to be insignificant. The sulfide reaction product of *p*-thiocresol and 2,4-di-(*p*-mercaptophenyl)-pentane was prepared by the reaction of the potassium mercaptide in refluxing alcohol for 5 hours with an equivalent amount of ω-bromoundecanoic acid followed by dilution with water and acidification. The product was recrystallized three times from Skellysolve B (b.p. 40–60°) and its molar extinction coefficient determined at 265 mμ and *p*H 10.

From 6.2 g. (0.05 mole) of *p*-thiocresol, 9.24 g. (0.11 mole) of potassium ethoxide in 200 ml. of alcohol and 13.5 g. (0.05 mole) of ω-bromoundecanoic acid was obtained 10 g. of product, m.p. 73–75°. Recrystallization gave an analytical sample, m.p. 77° (cor.). The molar extinction coefficient, ϵ, at 265 mμ and *p*H 10 was found to be 5.24×10^3, the ϵ-value of *p*-thiocresol at 265 mμ and *p*H 10 was 1.485×10^4. *Anal.*[16] Calcd. for $C_{17}H_{27}O_2S$: S, 10.43. Found: S, 10.69.

From 1.00 g. (0.00346 mole) of 2,4-di-(*p*-mercaptophenyl)-pentane and 1.2 g. (0.015 mole) of potassium ethoxide in 30 ml. of alcohol and 1.855 g. (0.0070 mole) of ω-bromoundecanoic acid was obtained 1.55 g. (68%) of the disulfide reaction product, m.p. 77–79°. Recrystallization gave an analytical sample, m.p. 81° (cor.). The ϵ-value at 265 mμ was 1.428×10^4; the ϵ value of 2,4-di-(*p*-mercaptophenyl)-pentane at 265 mμ was 3.072×10^4. *Anal.* Calcd. for $C_{39}H_{60}O_4S_2$: S, 9.76. Found: S, 9.97.

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[12] R. E. Basford and F. M. Huennekens, This Journal, **77**, 3873 (1955).

[13] Kindly supplied by F. Ortung of the Bakelite Division of Union Carbide and Carbon.

[14] P. D. Boyer, This Journal, **76**, 4331 (1954).

[15] R. G. Jones, *ibid.*, **69**, 2350 (1947).

[16] Analyses by Dr. F. Schwarzkopf, Woodside, New York, N. Y.

Method.—Two methods were used to follow the decrease in sulfhydryl concentrations when the appropriate thiol was treated with ω-bromoundecanoic acid.

For *p*-thiocresol and 2,4-di-(*p*-mercaptophenyl)-pentane the sulfide reaction product was prepared and its ultraviolet spectrum determined. Since the absorption peak for both free mercaptans occurs at 265 mμ, by following the decrease in absorbance at 265 mμ when the mercaptan is mixed with ω-bromoundecanoic acid solution the concentration of sulfhydryl groups at any time, *t*, is given by the relation

$$[\mathrm{SH}]_{\mathrm{t}} = \left[1 - \frac{OD_{\mathrm{rsh}} - OD_{\mathrm{t}}}{OD_{\mathrm{rsh}} - OD_{\mathrm{rsr}}}\right][\mathrm{SH}]_0$$

where OD_{rsh} is the initial optical density of the solution at 265 mμ due to the mercaptan alone and OD_{rsr} is the optical density of the solution at t_∞ or complete reaction when only the sulfide product is present (the ω-bromoundecanoic acid always was used in at least a threefold excess of that needed to react with all the sulfhydryl groups).

The second method employed for following the decrease in sulfhydryl concentrations of the hydrolyzed polymers in the alkylation reaction was the *p*-mercurichlorobenzoic acid method of Boyer.[14] The concentration of sulfhydryl groups in a solution of the hydrolyzed polymer was determined and this stock solution buffered at *p*H 10 was mixed with a freshly prepared solution of ω-bromoundecanoic acid also buffered at *p*H 10 to give 100 ml. of a solution containing at the start a sulfhydryl group concentration of 10 to 15 × 10^{-5} mole/l. and an ω-bromoundecanoic acid concentration of 40 × 10^{-5} mole/l. At 1-hour intervals for 5 hours, samples were withdrawn and treated with ten 3-ml. portions of *p*-mercurichlorobenzoic acid. A linear increase in absorbance at a wave length of 250 mμ (due to mercaptide formation) is found with increasing amounts of a sulfhydryl solution added to a fixed amount of *p*-mercurichlorobenzoic acid solution up to the equivalence point. A graphical plot of increase in absorbance at 250 mμ *versus* ml. of sulfhydryl solution added was sufficient to fix the unique sulfhydryl group concentration at hourly intervals for a total reaction time of five hours.

Results and Discussion

Oxidation of Thiols by Indophenol.—The mechanism for the oxidation of thiols by indophenol proposed by Basford and Huennekens involves a two-electron loss to give a sulfenic acid state from the mercaptan

$$\mathrm{RSH} \longrightarrow \mathrm{RS}^+ + \mathrm{H}^+ + 2\mathrm{e}^-$$

If this reaction is slow, then reaction with another RSH will produce a disulfide.

$$\mathrm{RS}^+ + \mathrm{RSH} \longrightarrow \mathrm{RSSR} + \mathrm{H}^+$$

In the reaction of the various thiols with indophenol carried out at *p*H 10, larger than stoichiometric ratios of thiol to dye were necessary to get measurable decoloration rates. This is not unexpected since the oxidation potential of the dye is dependent on the *p*H, becoming more negative at higher *p*H[17]; its resistance to reduction at *p*H 10, therefore, would be much greater than at *p*H 7. The initial reaction rates observed for these thiols at *p*H 10 was of the order of one-tenth as fast as those observed at *p*H 7 by Basford and Huennekens. This result is in line with their conclusion that the reactive form of the thiol is RSH and not RS^-.

The indicated uncertainties in the initial reaction velocities are indeterminate errors estimated[18] from relative errors in the experimental measurements. The largest error originated in the determination of the optical density at the end of the first 20 seconds when reduction was most rapid. This value was generally obtained from a reverse extrapolation of the smooth curve obtained on continued reduction.

TABLE I

REDUCED INITIAL RATE OF REDUCTION OF INDOPHENOL, V_0' BY VARIOUS THIOLS

Compound	Concn. of indophenol, *m*	V_0' (μm./ml.-min. × 10^{-3})
p-Thiocresol	2.61 × 10^{-5}	1.17 ± 0.10
Copolymer	2.66 × 10^{-5}	3.89 ± .20
Thioglycolic acid	2.64 × 10^{-5}	4.26 ± .20
2,4-Di-(*p*-mercaptophenyl)-pentane	2.61 × 10^{-5}	7.51 ± .30
Homopolymer	2.76 × 10^{-5}	8.88 ± .30

In the original work on this reaction, it was noted that 2,3-dimercaptopropanol showed a relatively fast rate of oxidation with a final ratio of dye reduced to thiol added of 1:2. The presence of two adjacent thiol groups was considered to facilitate the secondary reaction of a mercaptan group with an initially formed sulfenic acid state to produce a cyclic disulfide.

Molecular models show that in the case of 1,3-di-(*p*-mercaptophenyl)-alkanes, a cyclic disulfide is somewhat strainless since the two phenyl groups can lie in a parallel plane. As yet, we have not been able to isolate a pure monomeric disulfide, although it is clear that the suggestion is not unreasonable, even if part of the product is polymeric and part monomeric as we have found. The relatively fast rate of oxidation of 2,4-di-(*p*-mercaptophenyl)-pentane and the homopolymer therefore can thus be explained by this suggestion. Complete verification of this point must await the completion of a study of the cyclization of this and related dimercaptans currently in progress. *p*-Thiocresol, on the other hand, which oxidizes at a rate only about 15% as fast requires an intermolecular reaction to form a disulfide bond. The nearest sulfhydryl group to an initially formed sulfenic acid oxidation state evidently will be at a much greater distance in a dilute solution for *p*-thiocresol than if it were part of the same molecule as in the homopolymer and 2,4-di-(*p*-mercaptophenyl)-pentane. The equivalence of the sulfhydryl groups in *p*-thiocresol and in 2,4-di-(*p*-mercaptophenyl)-pentane in all other respects is shown in Fig. 3 where the final equilibrium concentration ratio of dye to thiol is reached by both at equivalent concentrations. Steric factors affecting the ease of oxidation of the sulfhydryl groups of the hydrolyzed copolymer are probably in an intermediate state. The positions of sulfhydryl groups along the polymer chain are no doubt closer than the distance at an equivalent concentration of *p*-thiocresol in a dilute solution; accordingly, the rate of oxidation found was intermediate. It is interesting that the rate for the copolymer was quite close to the oxidation rate found for thioglycolic acid, but a comparison on the basis of both containing free carboxyl groups does not seem justified at this time.

Alkylation Reactivity.—All reactions were run in *p*H 10 borate buffer at an initial concentration of ω-bromoundecanoic acid of 40 × 10^{-5} mole/l. Second-order plots for the reaction of *p*-thiocresol and 2,4-di-(*p*-mercaptophenyl)-pentane are shown

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(17) J. B. Conant, *Chem. Revs.*, **3**, 9 (1926).

(18) A. A. Benedetti-Pichler, *Ind. Eng. Chem., Anal. Ed.*, **8**, 376 (1936).

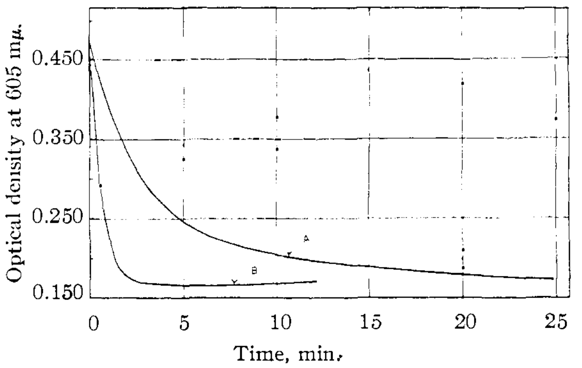

Fig. 3.—Rate of disappearance of 605 mμ peak of sodium 2,6-dichlorobenzeneoneindophenol at 3.46 ratio of SH/dye: A, *p*-thiocresol; B, 2,4-di-(*p*-mercaptophenyl)-pentane.

in Fig. 4. From the slope, *m*, of these plots the value of k_2 can be calculated.

The uncertainty in the values of k_2 was estimated from the fact that duplicate experiments agreed within 5%. Weighting of the sulfhydryl concentrations determined was made toward the first 3 hours of a 5-hour reaction period since the sulfhydryl concentration of a control solution at *p*H 10, in spite of all precautions to exclude traces of oxygen, fell off fairly rapidly after 5 hours to approximately 85% of its initial value. At 3 hours its value was 95+%.

TABLE II

DECREASE IN SULFHYDRYL CONCENTRATION FOR THE HYDROLYZED HOMOPOLYMER IN REACTION WITH ω-BROMOUNDECANOIC ACID[a] (FIG. 6)

Time, hr.	(SH) × 10^5, *m*	Reacn., %	k_2, l./mole-hr. × 10^2
0	10.56	0	....
1	8.54	19.2	5.56 ± 0.20
2	7.75	25.5	3.97 ± .20
3	7.40	29.9	3.03 ± .20
4	6.85	35.1	2.91 ± .20
5	6.65	37.1	2.48 ± .20

[a] Initial concentration of ω-bromoundecanoic acid 40.0 × 10^{-5}, temperature 30 ± 0.5°.

TABLE III

DECREASE IN SULFHYDRYL CONCENTRATION FOR THE HYDROLYZED COPOLYMER IN REACTION WITH ω-BROMOUNDECANOIC ACID[a] (FIG. 6)

Time, hr.	(SH) × 10^5, *m*	Reacn., %	k_2, l./mole-hr. × 10^2
0	13.35	0	....
1	11.60	13.0	3.64 ± 0.20
2	10.05	24.7	3.65 ± .20
3	9.35	30.0	3.17 ± .20
4	8.94	33.0	2.62 ± .20
5	8.56	35.8	2.41 ± .20

[a] Initial concentration of ω-bromoundecanoic acid 40.0 × 10^{-5} mole/l. temperature 30 ± 0.5°.

Reaction of *p*-thiocresol at an initial concentration of 14.60 × 10^{-5} mole/l. with ω-bromoundecanoic acid was approximately 63% complete in 5 hours with a k_2 value of 4.15 ± 0.15 × 10^2 l./mole-hr. 2,4-Di-(*p*-mercaptophenyl)-pentane at an initial sulfhydryl concentration 14.44 × 10^{-5} mole/l. showed 77% reaction in 5 hours with a k_2 value for each sulfhydryl group of 7.52 ± 0.30 × 10^2 l./mole/hr.

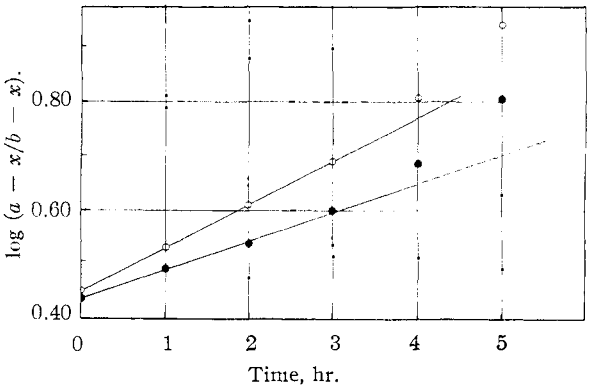

Fig. 4.—●, second-order plot for the reaction of *p*-thiocresol with ω-bromoundecanoic acid [RX]: a = [SH] = 14.60 × 10^{-5} mole/l.; b = [RX] = 40.00 × 10^{-5} mole/l.; ○, for reaction of 2,4-di-(*p*-mercaptophenyl)-pentane, same concn. for b, same temp., a = [SH] = 14.44 × 10^{-5} mole/l.

The relatively fast decrease in the velocity of reaction of the sulfhydryl group of the homopolymer with the long chain ω-bromoundecanoic acid seems to indicate that steric factors become rapidly important in the course of the alkylation reaction. The formation of one sulfide group along the polymer chain would be expected to hinder the possibility of collision of an adjacent sulfhydryl group and another molecule of alkyl halide. For 2,4-di-(*p*-mercaptophenyl)-pentane this steric factor is evidently not important since the rate constant for its reaction with the ω-bromoundecanoic acid did not decrease as the reaction progressed. For *p*-thiocresol, which also followed second-order kinetics, the second-order rate constant was, unexpectedly, lower than that of 2,4-di-(*p*-mercaptophenyl)-pentane although still faster than the average value found for the hydrolyzed polymer and copolymer. Further work is in progress with model compounds.

The decrease in the reaction rate constant on continued alkylation of the sulfhydryl groups of the hydrolyzed copolymer was not as pronounced as for the sulfhydryl groups of the hydrolyzed homopolymer. This would be expected since the distance between adjacent sulfhydryl groups is much greater and steric interaction between an initially formed sulfide group and the next sulfhydryl group would not be as important as in the homopolymer.

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