t-butyl hypochlorite, 2,3-epoxybutane with hydrochloric acid, 2,3-diacetoxybutane with hydrochloric acid, 3-chloro-2-butanol with thionyl chloride, with thionyl chloride and pyridine, with phosphorus trichloride, and with potassium hydroxide, is best accounted for on the basis of removal of one atom (or radical) with simultaneous attack at the back side of the carbon atom by a nucleophilic reagent. In some cases the attack is intramolecular, leading to the formation of a cyclic compound, in other cases the attack is intermolecular. All evidence is opposed to the concept of a carbonium ion as an intermediate.

When a carbon-to-chlorine bond possesses considerable ionic character, replacement of the chlorine atom by an electron donating reagent takes place by attack on the opposite side of the carbon atom with inversion, rather than at the front without inversion.

PASADENA, CALIFORNIA RECEIVED JUNE 18, 1941

[CONTRIBUTION FROM DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, SCHOOL OF MEDICINE, THE JOHNS HOPKINS UNIVERSITY]

o-Iodosobenzoic Acid, a Reagent for the Estimation of Cysteine, Glutathione, and the Substituent Sulfhydryl Groups of Certain Proteins[1]

BY LESLIE HELLERMAN, FRANCIS P. CHINARD[2] AND PAULINE A. RAMSDELL

During some thirty years past, there has appeared only occasional reference to the aryl iodoso, iodoxy, and related compounds,[3] a group of considerable theoretical interest. The importance of this group for valence studies is reflected in an electronic representation of iodosobenzene, $C_6H_5:\ddot{I}:\ddot{O}:$. Functioning as donor, this may add a proton, forming presumably the ion, $(C_6H_5IOH)^+$,[4] but there exist also the well-characterized dichloride, $(C_6H_5ICl)^+Cl^-$ and diacetate, $[C_6H_5I(OCOCH_3)]^+(OCOCH_3)^-$.

The better known simple iodoso compounds, functioning as "powerful" oxidizing agents, ordinarily are reduced *practically* irreversibly. This characteristic and the stability, observed in this study, of a slightly alkaline solution of certain salts of the readily available *o*-iodosobenzoic acid suggested a new approach to the troublesome problem of the estimation of cysteine and certain of its derivatives. This is based upon a reaction which has been found to proceed under proper conditions according to the process

$$2^-OOC{-}CH(NH_3^+){-}CH_2SH + C_6H_4(COO^-)IO \longrightarrow {}^-OOC{-}CH(NH_3^+){-}CH_2S \\ | \\ {}^-OOC{-}CH(NH_3^+){-}CH_2S + IC_6H_4COO^- + H_2O$$

The following is representative of the analytical method now developed.

Estimation of Cysteine Hydrochloride.—*o*-Iodosobenzoic acid, purchased on the market, was purified by solution in a small excess of chilled potassium hydroxide solution (2 *N*), filtration, and recovery by treatment of the alkaline solution with carbon dioxide.[5] The acid was washed with water and dried in a vacuum over phosphorus pentoxide. An approximately 0.02 *N* solution was prepared by addition to the reagent of a slight excess of normal potassium hydroxide and dilution to volume. Standardization was done iodometrically with the use of the relation $RIO + 2I^- + 2H^+ \rightarrow RI + I_2 + H_2O$.

A high-grade specimen of cysteine hydrochloride which had been shown by independent analysis to contain a little free cysteine and no more than a trace of cystine was made up to be *by weight*, 0.01039 *N* (uncor.). The sulfhydryl content was estimated as follows. To 5 ml. of molar phosphate buffer[6] of *p*H 7 (K_2HPO_4, 117.7 g. plus KH_2PO_4, 44.1 g., made to 1000 ml.) was added 10.00 ml. of *o*-iodosobenzoate (0.01812 *N*), after which 10.00 ml. of the cysteine hydrochloride was added to the mixture. After thirty seconds, there was added to this reaction mixture an acidified solution of potassium iodide (0.5 to 1 g. dissolved in 1.5 ml. of water and then treated with 5 ml. of *N* hydrochloric acid immediately before use). The liberated iodine was titrated at once with standard thiosulfate. In six successive titrations, the excess

(1) Aided by a grant from the Rockefeller Foundation Fluid Research Fund.

(2) Henry Strong Denison Scholar for 1940–41.

(3) Partial list of earlier references: V. Meyer and W. Wachter, *Ber.*, **25**, 2632 (1892); C. Willgerodt, *ibid.*, **25**, 3494 (1892); **26**, 1802 (1893); **27**, 2326 (1894); P. Askenasy and V. Meyer, *ibid.*, **26**, 1354 (1893); V. Meyer, *ibid.*, **26**, 2118 (1893); C. Hartmann and V. Meyer, *ibid.*, **27**, 1592 (1894).

(4) Iodoxybenzene hydrogen sulfate, $(C_6H_5IO_2H)^+(HSO_4)^-$, has been crystallized, I. Masson, *Proc. Roy. Inst. Gt. Brit.*, **30**, 99–122 (1937).

(5) *Cf.* A. S. Loevenhart and W. E. Grove, *J. Pharmacol. and Exp. Therap.*, **3**, 101 (1911).

(6) A. A. Green, THIS JOURNAL, **55**, 2331 (1933).

o-iodosobenzoate was found equivalent to 7.554 ± 0.004 ml. of 0.009588 *N* thiosulfate; the normality of the cysteine hydrochloride was, therefore, $[(10.00)(0.01812) - (7.554)(0.009588)]/10.00 = 0.01088$. This checked within 0.2% the results of an independent observer working with new standard solutions. Although no greater care is required than in general is needed in precise iodometric work, the precision attainable here is indeed surprisingly good.

The results were in acceptable agreement with the normality by weight when corrected on the basis of independent analyses of the cysteine hydrochloride used. For example, in four titrations of 10.0-ml. portions of the above cysteine hydrochloride solution against 0.02 *N* sodium hydroxide with chlor phenol red as indicator, the solution was found to be 0.009590 *N* in "combined HCl" (≏ cysteine *hydrochloride*). On this basis, it is shown readily by calculation that the solution should be 0.01064 *N* in "cysteine hydrochloride plus cysteine"; from Dr. T. S. Ma's determination of total sulfur in the dried sample (subs., 4.435, 4.866 mg.: $BaSO_4$, 6.720, 7.322 mg.; calcd. for $C_3H_8O_2NSCl$: 20.3; found 20.8, 20.7%), the normality, on the assumption that total sulfur equals HS-sulfur, would be 0.01062. Obviously, these are low rather than high values. On the other hand, the iodoso method may be expected to give somewhat high values (see below).

The value given by the iodoso method deviated by a few per cent. from the figures given by the less precise acid-iodometric methods, and was 1% lower than the result given by a painstaking assay with porphyrindine. The illustrative procedure is based upon numerous tests under varying conditions upon different preparations of cysteine salt.

Preparative

Isolation of Products.—Of *o*-iodosobenzoic acid, 1.32 g. was dissolved in 27 ml. of 0.2 *N* sodium hydroxide and filtered. The clear filtrate was added to 10 ml. of phosphate buffer (1 *M*, *p*H 7) and stirred mechanically, whereupon some of the acid precipitated; this was redissolved by the addition of the minimum quantity of strong potassium hydroxide. Cysteine hydrochloride, 1.58 g., dissolved in 20 ml. of water was added rapidly to the stirred buffered mixture, whereupon cystine slowly crystallized. This was collected, washed thoroughly with cold water, and dried in a vacuum desiccator over phosphorus pentoxide and flaked sodium hydroxide; yield, 1.09 g. (90%) of iodoso-free material. The cystine when dissolved in a small excess of hydrochloric acid and recrystallized by the cautious addition of ammonia water was recovered in 95% yield and was characterized by its chemical and physical properties; a second recrystallization rendered it halogen free.

The *p*H number of the filtrate from *l*-cystine was found upon test to be about 6.5. Roughly 20 ml. of *N* hydrochloric acid was added, and *o*-iodobenzoic acid was obtained as a flocculent precipitate. This was collected and dried. It was found by sodium fusion test to be sulfur-free, and melted at 161–162°; m. p. of mixture with authentic material, 162° (recorded m. p. of *o*-iodobenzoic acid, 162°).

Glutathione and Protein-SH Groups.—Reduced glutathione is the only cysteine-containing peptide to which the new method thus far has been applied. The details of procedure were entirely similar, and reproducibility of results equally good. The first step usually was allowed to proceed for one minute before addition of acidified iodide.

Excellent reproducibility was evident also in the results from attempts to estimate the substituent sulfhydryl groups of certain proteins after treatment ("denaturation") of the protein solutions under nitrogen in the presence of guanidine hydrochloride.[7] This application to proteins is in general urgently needed. The immediate requirement in this Laboratory was the desirability of precise estimation of the sulfhydryl groups of certain enzymes (*e. g.*, urease) in a study of the relation of enzymatic activity to such groupings. This will be presented elsewhere.[8] Exploratory experiments included the analysis of suitably recrystallized and dialyzed hen's egg albumin. To indicate precision, it may be stated that the agreement of results in numerous determinations upon 1-ml. portions of 3.58% albumin was within ±0.2%. Numerous determinations upon different preparations of ovalbumin gave results for the percentage of cysteine averaging 1.32%. This is on the assumption that there is substituted in this protein no source of HS-groupings other than cysteine. If so, the minimum average molecular weight of this albumin would be 36,700 on the basis of four cysteine residues per mole; 46,000, on the basis of five. The more reliable recent values by physical methods are stated[9] to be close to 45,000. The application of the iodoso method to proteins and other problems of biochemical interest will be treated in more detail in another place.

(7) J. P. Greenstein, *J. Biol. Chem.*, **125**, 501 (1938).

(8) *Cf.* L. Hellerman, *Cold Spring Harbor Symp. on Quant. Biol.*, **7**, 165–173 (1939); L. Hellerman, V. R. Deitz and F. P. Chinard, *J. Biol. Chem.*, **140**, scientific proceedings, p. 57 (1941).

(9) H. B. Bull, *ibid.*, **137**, 143 (1941).

Discussion: Limitations as Applied to Mercaptans in General.—The employment of unnecessarily large excesses of *o*-iodosobenzoate in the first step in the estimation of cysteine or glutathione leads to high results. Much work on this point has suggested to us that the oxidation probably involves two distinct concurrent reactions, one (predominant by far in the case treated here) the oxidation to the dithio configuration (cystine), and the second, oxidation to a sulfinic or sulfonic acid. In either case, the oxidation process may proceed through an intermediate ion or radical (RS^- or $R:\ddot{S}.$) or both. The second process is quite independent of the first, for cystine is not attacked by *o*-iodosobenzoate *at* *p*H 7 under the conditions used. If there be merit in the assumption that a radical $R:\ddot{S}.$ is the initial oxidation product and that this may dimerize rapidly to cystine, or alternatively, be oxidized further in the presence of excess iodoso reagent, it follows that the mercapto compound to be analyzed should be treated in a fairly dilute solution with a minimum workable excess of *o*-iodosobenzoate. Consequently, in the interest of accuracy if not precision, the application of the method demands orienting tests with respect to ascertainment of the excess required. For example, the excess used in our illustration is a convenient but not necessarily ideal minimum. The reaction may be carried out at 17–22°, and no advantage apparently is gained by the use of lower temperatures. The analytical operations may be carried out under nitrogen. The *p*H must be controlled; it will be shown elsewhere that at hydrion concentrations corresponding to $pH < 7$, even cystine begins to be oxidized by the acidified iodoso molecule $[(RIOH)^+(?)]$. Methionine is not oxidized by *o*-iodosobenzoate at *p*H 7 under the conditions specified, but in acid solution probably is converted to the sulfoxide.

Not alone on the counts of precision and convenience, but perhaps more significantly because of the non-reactivity of *o*-iodosobenzoate ion with most amino acids and with dextrose in solutions buffered at *p*H 7, the iodoso method appears to possess unique value. It will be recalled that important limitations in the use of porphyrindine[10] with proteins are found in the difficulty of its preparation, its instability, and its reactivity[11] with tyrosine. On the other hand, it appears that *o*-iodosobenzoate *under the conditions described* does not noticeably interact with dextrose, methionine, cystine, tyrosine, serine, tryptophan, proline, and oxyproline. The reagent, in common with porphyrindine, may be used in the estimation of 0.001 *N* ascorbic acid.

The estimation with iodoso compounds of such mercaptans as thioglycolic and thiosalicylic acids has not yet been developed fully. A proper control of analytical or preparative details will require further study. With respect to such mercaptans as are found in petroleum, it is an interesting question whether the iodoso series will be found of analytical value. It is anticipated, however, that they will be found of interest in certain investigative procedures involving generally the oxidation of mercaptans, since iodoso compounds of varying physical properties are readily synthesized.

Summary

In this preliminary paper, it is brought out that the aryl iodoso compounds readily oxidize mercaptans; there are presented, in particular, the details of a method of some precision for the estimation of cysteine with the use of standard *o*-iodosobenzoate. In addition, the preparative details of oxidation of cysteine to cystine by *o*-iodosobenzoate ion are given. Reference is made also to the estimation of glutathione and the substituent sulfhydryl groups of ovalbumin and of urease. The limitations of the method are discussed briefly.

BALTIMORE, MD. RECEIVED JULY 18, 1941

(10) For references, see C. C. Porter and L. Hellerman, THIS JOURNAL, **61**, 754 (1939).

(11) E. Brand and B. Kassell, *J. Biol. Chem.*, **133**, 437 (1940).