pressure to yield bis(2-aminoethyl) diselenide dihydrochloride (8.8 g, 55%; mp 179° dec). The compound had an infrared spectrum identical with that of an authentic sample.[14]

4,4'-Diselenodibutyric Acid (IV).—The selenium–magnesium reagent (0.1 equiv) was heated for 20 hr under reflux with butyrolactone (10.1 g, 0.12 mole). The solvent was then evaporated, water added, and the acidified solution extracted with ether. The yellow ether solution was reextracted with sodium hydroxide and the 4,4'-diselenodibutyric acid (11.0 g, 66%; mp 84–86°) precipitated by addition of hydrochloric acid. The dried product was recrystallized from carbon tetrachloride to give platelets, mp 88°, which had an infrared spectrum identical with that of an authentic sample.

α,α'-Diselenodi-o-toluic Acid (V).—Phthalide (13.4 g, 0.1 mole) was heated for 20 hr under reflux and stirring with the selenium–magnesium reagent (from 0.1 g-atom of Se). The clear supernatant liquid was then decanted and discarded and the dark solid was shaken with water (300 ml) and hydrochloric acid (30 ml of 12 *N*) until it was thoroughly dispersed. The crude product was collected by filtration, dissolved in 2 *N* aqueous sodium hydroxide, and aerated until precipitation of selenium appeared complete. Elemental selenium was removed by filtration with Celite and the clear, orange filtrate acidified with hydrochloric acid. The yellow precipitate of α-α'-diselenodi-o-toluic acid (18 g, 82%; mp 215–218°) was collected and dried over phosphorous pentoxide. For analysis a sample was recrystallized from ethanol. The compound dissolves very slowly in boiling ethanol and crystallizes over a period of days; the melting point was not changed by this treatment.

Anal. Calcd for $C_{16}H_{14}O_4Se_2$: C, 44.88; H, 3.30; Se, 36.88. Found: C, 44.77; H, 3.47; Se, 35.14.

Discrepancy in the selenium values necessitated the preparation of the two derived compounds given below.

2-Selenophthalide (VI).—α,α-Diselenodi-o-toluic acid (10.7 g, 0.05 mole) and 50% aqueous hypophosphorous acid (8 ml) were heated together in a 250-ml erlenmeyer flask on an electric hot plate until the water had evaporated and a sudden vigorous reaction set in. The yellow product was dissolved in ether and passed through a column of activated alumina (Woelm, neutral, activity grade I). The colorless effluent was evaporated to dryness and the residue crystallized from petroleum ether (bp 36–55°) to give colorless scales of 2-selenophthalide (6.7 g, 62%), mp 56.5–58° (lit.[15] mp 58°).

Anal. Calcd for C_8H_6OSe: C, 48.75; H, 3.07; Se, 40.06. Found: C, 48.99; H, 3.10; Se, 40.09.

N,N'-n-Butyl-α,α-diselenodi-o-toluamide (VII).—2-Selenophthalide (2.0 g, 0.01 mole) was dissolved in ethanol (25 ml) and *n*-butylamine (2 ml) added. The reaction was allowed to proceed in an open flask overnight; water was then added until crystallization set in to give yellow needles of VII (2.7 g, quantitative yield), mp 144–146°.

Anal. Calcd for $C_{24}H_{32}N_2O_2Se_2$: C, 53.53; H, 5.99; N, 6.20. Found: C, 53.45; H, 5.90; N, 6.16.

Registry No.—I, 1482-82-2; III, 3542-13-0; IV, 14362-48-2; V, 10352-20-2; VII, 14310-08-8; bis(methoxymagnesium) diselenide, 14310-09-9.

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Methods in Selenium Chemistry. III.[1,2] The Reduction of Diselenides with Dithiothreitol

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The interaction of aliphatic diselenides and dithiothreitol in aqueous solution leads to the quantitative formation of selenols. A new ultraviolet absorption peak in the 243–253-mμ range is attributed to the aliphatic selenol anion.

Selenols are readily oxidized to the corresponding diselenides upon exposure to atmospheric oxygen. In addition they are extremely powerful hydrogen donors capable of reducing disulfides, sulfoxides, and aromatic azo compounds[1] as well as other mild oxidizing agents such as 2,4-dichlorophenolindophenol.[4] Chemical or biological work with dilute solutions of organic selenols must, therefore, be carried out with the strict exclusion of air and this limitation has frequently prevented the accumulation of accurate data. Diselenides, on the other hand, are relatively stable compounds that can be prepared conveniently by the oxidation of selenols[5] or from other suitable intermediates, such as selenocyanates,[5] or selenosulfate Bunte salts.[6] In aqueous solution diselenides are conveniently converted to selenols by reaction with sodium borohydride[6] under alkaline conditions, or with hypophosphorous acid[1] if an acidic medium is preferred. In both cases the progress of the reaction can be followed by measuring the disappearance of the low intensity ultraviolet absorption peak near 300 mμ which is characteristic of aliphatic diselenides[7] or, in preparative work, by observing the disappearance of the yellow color in the solution. Quantitative determinations of diselenides based on ultraviolet absorption are, however, quite unsatisfactory if the molar concentration is lower than 10^{-3}. A more sensitive assay for diselenides is based on the observation[8] that iodine in the presence of bicarbonate buffer converts diselenides into the corresponding seleninic acids and that this reaction is reversed upon acidification of the solution, allowing the liberated iodine to be determined by standard procedures. A variation of this latter method has been used to detect small amounts of selenols, diselenides, and monoselenides on paper and thin layer chromatograms.[9] The recent observation[10] that dithio-

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threitol[11,12] imparts catalytic activity to seleno coenzyme A diselenide in the acetylation of hydroxylamine by ATP and acetate in the presence of acetyl coenzyme A synthetase from yeast[13] indicated that reduction of the inactive diselenide to the active selenol form had taken place. Reduced glutathione imparted only marginal activity to this system.

Since its recent introduction,[11] Cleland's reagent has found wide applications in the protection of sulfhydryl groups in biochemical reactions. The main advantages of both dithiothreitol (DTT) and the related dithioerythritol (DTE) over previously used thiols, such as mercaptoethanol, are their extremely low redox potentials (−0.332 v at pH 7 for DTT), the high stability to atmospheric oxygen even in aqueous solution, and, for aesthetic considerations, the virtual absence of any objectionable odor. When it was attempted to determine the rate at which DTT reacted with 2-trimethylammoniumethyl diselenide (choline diselenide)[6] in aqueous solution at pH 7.6, it was found that the diselenide absorption disappeared nearly instantaneously and a peak of high intensity appeared at about 250 mμ, partly masked by the high end absorption of the original solution. When access of air was maintained, the new absorption peak remained stable for varying periods of time depending on the concentrations of DTT originally employed here. Then a slow decay was noted and the final spectrum of the solution appeared to be the sum of the absorptions of the original diselenide and of *trans*-4,5-dihydroxy-1,2-dithiane, the oxidation product of DTT. Since the possible reduction product cholineselenol[4] has a pK of 4.77, it must be completely ionized at the pH the reaction was run. The new peak was then due to either the selenol anion or to a product formed by interaction of DTT with the diselenide. Pure cholineselenol iodide[4] is rapidly oxidized in dilute aqueous solutions making spectral measurements very difficult; a rapidly decreasing absorption maximum at 243 mμ was, however, observed in neutral or alkaline solution. This peak disappeared instantaneously upon acidification to pH 2 or below. To isolate the spectrum of the newly formed chromophore, reactions of a number of diselenides with DTT were carried out in split compartment cells under difference spectroscopy conditions. All reactions were run in Tris buffer at pH 7.6, since a higher pH gave a much increased end absorption due to ionization of DTT–thiol groups, making it difficult to balance the slit and base line of the instrument. The data given in Table I show that DTT reacted with all compounds tested with the exception of 1,2-diselenolane-4-carboxylic acid. In all these cases a greatly increased ultraviolet absorption in the 243–251-mμ range resulted.

The conclusion that absorption was due to the selenol anion is strongly supported by titration data. Here compounds I–VI were titrated with 0.01 N NaOH in the presence and absence of dithiothreitol. The alkali consumption due to excess reducing agent was determined separately. After correction for this amount (about 1.5% of the total at pH 7.6), the titrations showed that in each case formation of a moderately strong acid (2 equiv for each mole of diselenide employed) had taken place and that at pH 7.6 the new acids were fully ionized. Similar titrations of diselenides carrying acidic side chains were carried out on the easily soluble 2,2-diselenodiacetic acid (VIII) and on barium selenopantethine-4′-phosphate (XI). In these cases both the ultraviolet absorption and the alkali consumption at pH 7.6 were found to be proportionately less than the figures found for the diselenides carrying amino groups. Ultraviolet measurements at pH values much higher than pH 7.6, as well as titrations beyond that value, are difficult to assess quantitatively, since ionization of DTT–thiol groups occurs in that range. The newly found absorption peak of aliphatic selenol anions appears to correspond in position and intensity to a well-known, but rather sparsely documented, absorption of aliphatic mercaptide ions in the 230–240-mμ region. Thus, alkaline solutions of *n*-butylmercaptan have an absorption peak of ϵ_{240}^{max} 5500[14] and sodium sulfide absorbs at ϵ_{232}^{max} 7500.[14] In acidic solution these absorptions are, essentially, nonexistent. The differential absorption of thiols and their anions has been employed widely in spectrophotometric determinations of acid dissociation constants for a number of sulfhydryl compounds (see, *e.g.*, ref 15–20) but details of the actual spectra so used appear to be lacking in nearly every instance. This is, presumably, due to the considerable experimental uncertainty which afflicts work with thiols under alkaline conditions, where even traces of atmospheric oxygen may interfere with exact determinations of extinction coefficients. The position of the absorption maxima assigned to some RS^- species does, also, appear to shift with varying pH in a number of sulfhydryl compounds. Thus, a range of 238–230 mμ is given[15] for 2-aminoethanethiol, cysteine, homocysteine, glutathione, and related compounds as the pH of the solution is changed from 12 to about 8.

The nature of the electronic transitions which give rise to these thiol anion absorptions has never been thoroughly investigated. It has been pointed out,[14] however, that a curious coincidence exists between these and the main diagnostic peak of alkanethiolesters in the 232–235-mμ region. A similar relationship does exist between selenol anion and selenolesters. Thus, Se-acetyl-N-succinoylselenocysteamine has ϵ_{253}^{max} 2790 with a very strong end absorption at the limit of the instrument (185 mμ). The corresponding Se-benzoyl-N-succinoylselenocysteamine absorbs at ϵ_{242}^{max} 12,500 and ϵ_{286}^{max} 5800 plus an inflection at 305 mμ and the spectrum of the only known aliphatic selenolactone, γ-selenobutyrolactone,[1] again has two peaks at ϵ_{197}^{max} 5500 and ϵ_{258}^{max} 3100.

Considerable difficulties will, therefore, be encountered when selenolester hydrolyses are to be monitored under neutral or alkaline conditions by observing changes in ultraviolet absorption in the region of 250 mμ, as noted earlier[14] for thiol esters. Hydrolyses and

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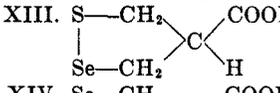
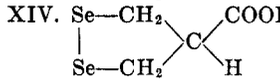
TABLE I
REDUCTION OF ALIPHATIC DISELENIDES WITH DITHIOTHREITOL

			RSeH at pH 7.6		
$(RSe)_2$	Salt form	Ref	λ_{max}, mμ	ϵ_{max}	OH^- equiv consumed
I. $H_2N-CH_2CH_2-$	HCl	*a*	243	7050	1.03
II. $CH_3NH-CH_2CH_2-$	HCl	*a*	243	7850	1.05
III. $(CH_3)_2N-CH_2CH_2-$	HCl	*a*	243	7400	1.00
IV. $(CH_3)_3N^+-CH_2CH_2-$	I^-	*a*	243	7200	1.00
V. $(CH_3)_2N-CH_2CH_2CH_2-$	HCl	*b*	247	6950	0.93
VI. $(CH_3)_3N^+-CH_2CH_2CH_2-$	I^-	*b*	244	7500	0.97
VII. $HOOC-CH_2CH_2CONH-CH_2CH_2-$			247	7000	...
VIII. $HOOC-CH_2-$		*c*	251	4100	0.45
IX. $HOOC-CH_2CH_2-$		*d, e*	250	4350	...
X. $HOOC-CH_2CH_2CH_2-$		*e, f*	250	4300	...
XI. Se-Pantethine-4′-phosphate	Ba^{2+}	*g*	247	3600	0.39
XII. Seleno coenzyme A	Li^+	*g*	243	7100	...
XIII. 		*h*	253	1250	...
XIV. 		*i*	No reaction		

[a] See ref 6. [b] See ref 4. [c] O. Behagel and M. Rollmann, *Ber*, **62**, 2698 (1929). [d] H. J. Backer and W. van Dam, *Rec. Trav. Chim.*, **49**, 485 (1930). [e] A. Fredga, Doctoral Dissertation, University of Uppsala, Sweden, 1935. [f] See ref 1. [g] See ref 9. [h] G. Bergson and A. Biezais, *Arkiv Kemi*, **18**, 143 (1961). [i] G. Bergson, *ibid.*, **19**, 195 (1962).

aminolyses of selenobenzoyl compounds[9,21-23] can, however, be followed at 285 mμ without undue interference. The discovery of an ultraviolet absorption peak for aliphatic selenol anions combined with the facile reduction of diselenides by dithiothreitol should prove valuable in determinations of dissociation constants and oxidation–reduction potentials as well as in biological work with organic selenium compounds.

Experimental Section

Ultraviolet spectra were determined on a Model 15 Cary spectrophotometer; matched dual compartment cells for difference spectroscopy were purchased from the Pyrocell Manufacturing Co., Westwood, N. J.; dithiothreitol was purchased from Calbiochem, Los Angeles, Calif.; titrations were carried out on a TTTl Titrigraph automatic titrator from Radiometer, Copenhagen, Denmark; synthetic procedures for most of the diselenides used here have appeared elsewhere; some new compounds are described below.

Difference Spectroscopy.—Stock solutions of diselenides ($\sim 10^{-4}$ *M*) and of DTT (2 to 5 × 10^{-4} *M*) were made up in 0.1 *M* Tris buffer [from tris(hydroxymethyl)aminomethane and hydrochloric acid] at pH 7.6. Equal volumes (usually 1.0 ml) of diselenide and reducing agent solutions were pipetted into adjacent compartments of difference spectroscopy cells; two such cells were placed into sample and reference beams of a double-beam spectrophotometer, and the base line balance was verified over a spectral range of 350–220 mμ. The solutions in the sample beam were mixed by several rapid inversions of the capped cell, the difference spectrum was determined immediately and again after an interval of 5–10 min to verify stability of the resulting spectrum. The observed absorption maxima and extinction coefficients (based on equivalents of selenol produced) are given in Table I.

N,N′-Disuccinoylselenocystamine (VII).—Succinic anhydride (10.0 g, 0.1 mole) was added in small portions to a well-stirred aqueous (70 ml of water) solution of selenocystamine dihydrochloride[24,25] (15.95 g, 0.05 mole) containing sodium hydrogen carbonate (21 g, 0.25 mole). Stirring was continued until the evolution of carbon dioxide ceased; then 6 *N* hydrochloric acid was added until pH 1 was obtained. The copious precipitate (21.5 g, 97% of theory) was collected by filtration and recrystallized from hot water to give VII as a fine yellow powder, mp 150–151°.[26]

Anal.[27] Calcd for $C_{12}H_{20}N_2O_6Se_2$: C, 32.30; H, 4.52; N, 6.28; Se, 35.39. Found: C, 32.52; H, 4.21; N, 6.48; Se, 35.67.

Se-Acetyl-N-succinoylselenocysteamine.—In an atmosphere of pure nitrogen, a solution of N,N′-disuccinoylselenocystamine (4.5 g, 0.01 mole) was dissolved in methanol (30 ml) and reduced by addition of solid sodium borohydride (about 1.0 g) until the solution appeared colorless. Acetic anhydride (70 ml) was then added slowly with stirring and cooling and its excess was destroyed after 10 min by adding more methanol. All solvents were then evaporated and the oily residue was dried on an oil pump. The residue was extracted continuously with anhydrous ether and the crystalline product (3.7 g, 69%), mp 108–110°, was analyzed after one crystallization from ether, which did not change the physical constants.

Anal. Calcd for $C_8H_{13}NO_4Se$: C, 36.10; H, 4.92; N, 5.26; Se, 29.67. Found: C, 36.15; H, 5.19; N, 4.90; Se, 29.97.

Ultraviolet absorption (H_2O) was at ϵ_{253}^{max} 2790.

Se-Benzoyl-N-succinoylselenocysteamine.—N,N′-Disuccinoylselenocystamine (4.5 g, 0.01 mole) was suspended in water (50 ml) and reduced by addition of solid sodium borohydride (up to 1.0 g) until a colorless solution resulted. Sodium bicarbonate (2.0 g) was then added and the solution was stirred with benzoyl chloride (3.0 g, 0.021 mole) until the smell of the acid chloride had disappeared. The solution was then acidified with 2 *N* hydrochloric acid and the precipitated solid (5.1 g, 79%) was recrystallized from very dilute ethanol to give colorless needles, mp 113–115°.

Anal. Calcd for $C_{13}H_{15}NO_4Se$: C, 47.57; H, 4.61; N, 4.27; Se, 24.05. Found: C, 47.66; H, 4.25; N, 4.20; Se, 24.45.

The ultraviolet spectrum (H_2O) was determined at ϵ_{242}^{max} 12,500; ϵ_{286}^{max} 5800.

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