and 1705 cm^{-1}; *m/e* 442 (<1 %), base peak *m/e* 382 (M−60), 367, 296, 281, 261, 255, 228, 213; τ 9.32 (s, 3H, C−18), τ 8.97 (s, 3 H, C−19), τ 8.91 (d, J ~7 cps, 9H, C−21, C−26, C−27), τ 7.97 (s, 3H, acetate), τ ~5.4 (m, 1H, C−3), and τ ~4.6 (diffuse triplet, 1H, C−6).

24-Oxocholesterol was obtained by hydrolysis of 24-oxocholesteryl acetate with 5 % KOH in methanol at room temperature overnight, m.p. 133−134°C after crystallization from methanol, mixed with synthetic material (see below) m.p. 135−137°C; ν_{max} (KBr) 1710 cm^{-1}; M^+ found 400.3356, calc. for $C_{27}H_{44}O_2$ 400.3341, *m/e* 400 (100 %), 385 (11 %), 382 (28 %), 367 (10 %), 315 (16 %), 314 (30 %), 299 (9 %), 289 (18 %), 281 (10 %), 273 (9 %), 271 (15 %), 255 (14 %), 246 (7 %), 231 (9 %), 229 (9 %), 213 (20 %), 71 (40 %), 43 (84 %).

24-Oxocholesterol from fucosterol. Fucosterol (42 mg) was treated with osmium tetroxide (25 mg) in dry benzene and a few drops of pyridine at room temperature overnight. The product was refluxed with sodium sulfite (0.25 g) in alcohol for 2 h. After filtration through Celite, the solution was evaporated and the glycol (38 mg) was oxidized with periodic acid (34 mg) in ethanol and pyridine at room temperature overnight. Extraction with ether gave 34 mg of 24-oxocholesterol, m.p. 137−138°C after crystallization from methanol; ν_{max} (KBr) 1710 cm^{-1}; *m/e* 400 (100 %), 385 (10 %), 382 (23 %), 367 (10 %), 315 (16 %), 314 (25 %), 299 (9 %), 289 (16 %), 281 (8 %), 273 (9 %), 271 (14 %), 255 (14 %), 246 (6 %), 231 (10 %), 229 (10 %), 213 (19 %), 71 (42 %), 43 (96 %); τ 9.32 (s, 3H, C−18), τ 8.98 (s, 3H, C−19), τ 8.91 (d, J ~7 cps, 9H, C−21, C−26, C−27), τ 8.33 (s, 1H, OH), τ ~6.5 (m, 1H, C−3), τ ~4.65 (diffuse triplet, 1H, C−6).

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Note added in proof. After this note had been submitted for publication we became aware of a short communication by Knights, B.A. *Phytochemistry* **9** (1970) 903. Dr. Knights reported *inter alia* the presence of 24-oxocholesterol in the brown alga *Ascophyllum nodosum*, but suggested that it is an artefact arising from fucosterol by aerial oxidation during storage. Although in our case we cannot with certainty exclude this possibility, we think it unlikely in view of the great care taken to avoid uncontrolled oxidation during the various operations.

1. Heilbron, I. M., Parry, E. G. and Phipers, R. F. *Biochem. J.* **29** (1935) 1376.
2. Black, W. A. P. and Cornhill, W. J. *J. Sci. Food Agr.* **2** (1951) 387.
3. Nes, W. R., Castle, M., McClanahan, J. L. and Settine, J. M. *Steroids* **8** (1966) 655.
4. Bergmann, J., Lindgren, B. O. and Svahn, C. M. *Acta Chem. Scand.* **19** (1965) 1661.
5. *Elsevier's Encyclopedia of Organic Chemistry*, Vol. 14, Supplement, New York-Amsterdam 1954, p. 1793 s.
6. Knights, B. A. *J. Gas Chromatog.* **5** (1967) 273.
7. Bergmann, W. and Dusza, J. P. *Ann.* **603** (1957) 36.

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The Synthesis of a Mixed Disulfide of Glutathione and 3-Carboxy-4-nitrobenzenethiol ("Reduced Ellman's Reagent")

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We have previously prepared and studied mixed disulfides of glutathione and naturally occurring aliphatic thiols.[1-4] In these investigations, as well as in studies made in other laboratories, it has been found that among a number of aliphatic disulfides, only the mixed disulfide of coenzyme A and glutathione gives an activity with glutathione reductase, which exceeds 1 % of the activity obtained with glutathione disulfide.[5,6] Since very little is known about the interaction between glutathione reductase and its disulfide substrate, it is of interest to obtain glutathione sulfenyl derivatives other than glutathione

disulfide, to investigate the effect of structure on the enzymatic activity.

It was decided to attempt the synthesis of the mixed disulfide of glutathione and 3-carboxy-4-nitrobenzenethiol ("reduced Ellman's reagent" [7]), not only to investigate its possible function as a substrate for glutathione reductase but also to test its effect on other glutathione-linked enzymes, such as *S*-lactylglutathione lyase and the enzyme catalyzing thiol-disulfide interchange between glutathione and the mixed disulfide of cysteine and glutathione (see Ref. 8).

The present communication describes a procedure, which consistently gives a yield of about 10 % of this mixed disulfide.

The substrate function of the new mixed disulfide for glutathione reductase and the enzyme catalyzing thiol-disulfide interchange will be described elsewhere. *S*-Lactylglutathione lyase, which is inhibited by disulfides (see Ref. 9, and papers cited therein), was reversibly inhibited by 0.3 mM of the mixed disulfide.

Experimental. Synthesis. 5,5'-Dithiobis(2-nitrobenzoic acid) (150 mg, 380 nmoles) was suspended in 10 ml of deionized water and dissolved by automatic titration (Radiometer Titrator TTT1c and Titrigraph SBR 2c equipped with a 10 ml syringe) with 0.5 M NaOH. To this solution 50 µl aliquots of glutathione (100 mg, 320 nmoles, dissolved in 1 ml of water) were slowly added with stirring. The pH was automatically readjusted to pH 6.5 by the titrator. An orange colour developed due to the liberation of one 3-carboxy-4-nitrobenzenethiolate ion for each glutathione molecule added.

Purification. The reaction mixture was applied to a DEAE-Sephadex A-25 column (2 × 10 cm, Cl^- form, packed in water) after the last addition of glutathione. The column was washed with 50 ml of water, and the components of the reaction mixture were then eluted by a linear NaCl-gradient (0 – 0.5 M; total volume: 1600 ml). The compounds eluted were (in order): two minor components, glutathione disulfide, the mixed disulfide, an unidentified component, and 3-carboxy-4-nitrobenzenethiolate and residual 5,5'-dithiobis(2-nitrobenzoate). The mixed disulfide appeared in the effluent at an ionic strength of about 0.16 M and was clearly resolved from the other components. It was identified by being strongly UV-absorbing, ninhydrin-positive, and giving a yellow product (3-carboxy-4-nitrobenzenethiolate) upon reduction with an excess of thiol. Furthermore, it was homogeneous in two electrophoretic systems (pH 1.9 and 4.0), showing no UV-absorbing, ninhydrin-positive, or fluoresceine mercuric acetate-positive [10] impurities. Its electrophoretic mobility was also consistent with the identification. The pooled fractions of the ion-exchange chromatography (134 ml) were analyzed quantitatively by reducing an aliquot with a large excess of glutathione. The liberated 3-carboxy-4-nitrobenzenethiolate was determined spectrophotometrically at 412 nm (*cf.* Ref. 7). The yield of the mixed disulfide was 28 nmoles, 9 %.

The solution containing the mixed disulfide can be stored for at least a week in a refrigerator without detectable decomposition. However, to prevent microbial growth and disulfide dismutation it is advisable to remove the water in a rotatory evaporator.

It has not, as yet, been possible to find a suitable method for removing NaCl from the product. Gel filtration on Sephadex G-10 or chromatography on BioRad ion-retardation resin cannot be used due to the strong adsorption of the mixed disulfide to the chromatographic bed. However, the product can be used in experiments in which the NaCl does not interfere.

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1. Eriksson, B. *Acta Chem. Scand.* **20** (1966) 1178.
2. Eriksson, B. and Eriksson, S. A. *Acta Chem. Scand.* **21** (1967) 1304.
3. Mannervik, B. and Nise, G. *Arch. Biochem. Biophys.* **134** (1969) 90.
4. Mannervik, B. Fil.dr. thesis, Stockholm 1969.
5. Ondarza, R. N. and Martínez, J. *Biochim. Biophys. Acta* **113** (1966) 409.
6. Chang, S. H. and Wilken, D. R. *J. Biol. Chem.* **241** (1966) 4251.
7. Ellman, G. L. *Arch. Biochem. Biophys.* **82** (1959) 70.
8. Eriksson, S. A. and Mannervik, B. *FEBS Letters* **7** (1970) 26.
9. Ekwall, K. and Mannervik, B. *Arch. Biochem. Biophys.* **137** (1970) 128.
10. Karush, F., Klinman, N. R. and Marks, R. *Anal. Biochem.* **9** (1964) 100.

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