(1) thiodione is intensely colored, evidence of the presence of a quinone ring (the above bisulfite addition compound does not absorb above 385 $m\mu$); and (2) the yield of thiodione was found to depend on the presence or absence of air, whereas formation of the above structure should be independent of the availability of oxygen.

Inspection of a molecular model of thiodione (Fig. 7) shows that hydrogen bonding can take place between the quinone oxygens and either the amino nitrogens or the carboxylic oxygens of the glutathione moiety and may thus stabilize the molecule. The redox potential of thiodione precludes involvement of the quinone oxygens in covalent bonding, resulting in ring closure, as was postulated by Kuhn and Beinert (1944) for the complex formed between benzoquinone and cysteine. The fact that reduced menadione, despite its somewhat lower redox potential, can exist in the presence of thiodione provides a further indication of stabilization of the quinone oxygens of thiodione by hydrogen bonding or steric hindrance.

REFERENCES

Bongartz, J. (1888), *Ber. 21*, 483.

Carmack, M., Moore, M. B., and Balis, M. E. (1950), *J. Am. Chem. Soc. 72*, 844.

Clark, W. M. (1960), Oxidation-Reduction Potentials of Organic Systems, Baltimore, The Williams & Wilkins Co., p. 376.

Fieser, L. F., and Fieser, M. (1956), Organic Chemistry, Boston, Heath & Co., p. 718.

Fieser, L. F., and Turner, R. B. (1947), *J. Am. Chem. Soc. 69*, 2335.

Hart, E. J. (1951), *J. Am. Chem. Soc. 73*, 68.

Katchalski, E., Benjamin, G. S., and Gross, V. (1957), *J. Am. Chem. Soc. 79*, 4096.

Kuhn, R., and Beinert, H. (1944), *Ber. 77B*, 606.

Michaelis, L., Schubert, M. P., and Smythe, C. V. (1936), *J. Biol. Chem. 116*, 587.

Mulliken, R. S. (1952), *J. Am. Chem. Soc. 74*, 811.

Nickerson, W. J., Falcone, G., and Strauss, G. (1960), *Fed. Proc. 19*, 35.

Powning, R. F., and Irzykiewicz, H. (1960), *Australian J. Biol. Sci. 13*, 59.

Schubert, M. (1947), *J. Am. Chem. Soc. 69*, 712.

Snell, J. M., and Weissberger, A. (1939), *J. Am. Chem. Soc. 61*, 450.

Strauss, G., and Nickerson, W. J. (1961), *J. Am. Chem. Soc. 83*, 3187.

Whitby, L. G. (1952), *Biochem. J. 50*, 433.

Studies on Quinone-Thioethers. II. Photochemical and Hydrolytic Cleavage of Thiodione[*]

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Anaerobic illumination with visible light of an aqueous solution of thiodione (2-methyl-3-glutathionyl-1,4-naphthoquinone) results in development of a burgundy color and a drop in potential. These effects result from the cleavage of thiodione with the formation of 2-methyl-3-mercapto-1,4-naphthoquinone (burgundy color) and dihydro-thiodione (lowered potential). In contrast, alkali-catalyzed cleavage of thiodione, in the dark, yields phthiocol and glutathione. The fact that phthiocol results from alkaline hydrolysis of thiodione (a quinone-thioether), and also from saponification of the acetone-soluble fat of human tubercle bacilli, suggests that the natural precursor of phthiocol may be a 2-methyl-naphthoquinone bound *via* a thioether linkage.

In the course of a study of the properties of thiodione (2-methyl-3-glutathionyl-1,4-naphthoquinone; Nickerson *et al.*, 1963), the substance was observed to be photolabile, as evidenced by rapid formation of a burgundy color in aqueous solutions exposed to visible light. Photochemical activity of quinone-thioethers has not been reported previously; however, inactivation and decomposition of the related vitamin K_1 by light (Almquist, 1936, 1937; MacCorquodale *et al.*, 1939; Ewing *et al.*, 1943) is well known. Therefore, photodecomposition of thiodione was studied, and a degradation product of the quinone moiety was identified.

EXPERIMENTAL

Materials.—The synthesis of thiodione has been described in the first paper of this series (Nickerson *et al.*, 1963). All substances used as chromatographic reference standards were tested and recrystallized if necessary until chromatographically pure. Nitrogen, from the Matheson Co., of 99.99% purity, was used to flush solutions to make them air-free.

The burgundy photodecomposition product of thiodione, later identified as 2-methyl-3-mercapto-1,4-naphthoquinone (prepared according to Nickerson, unpublished, and referred to as mercapto-menadione), was isolated from an illuminated thiodione solution as follows: 10 mg of thiodione in 50 ml water was illuminated by a 375-watt photoflood lamp at a distance of 10 cm. Nitrogen was flushed through the solution, which was kept cool under running water. At the end of 4 hours' illumination, the solution, now deep red and turbid, was acidified with HCl, whereupon it became pale yellow. It was exhaustively extracted with chloroform. When a sample of the pale yellow chloroform extract was shaken with aqueous NaOH, the characteristic burgundy color of illuminated thiodione appeared in the aqueous phase. The bulk of the chloroform extract was evaporated to dryness and yielded 2 mg of a dark reddish-brown product.

Potential Measurements.—For simultaneous measurement of potential and optical density during anaerobic illumination of thiodione solutions, an evacuable cell fitted with platinum and calomel electrodes (Strauss and

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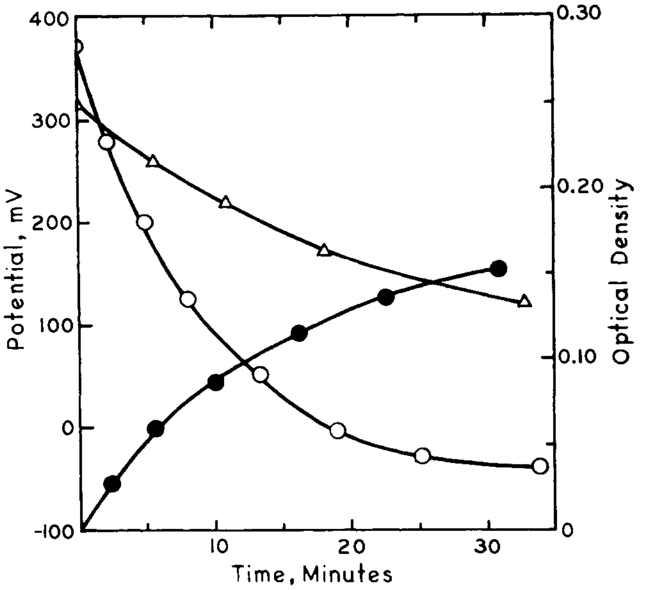


FIG. 1.—Changes in potential and optical density during anaerobic illumination of thiodione (5×10^{-5} M) in *p*H 7 phosphate buffer. ○, potential of platinum electrode relative to normal hydrogen electrode. △, optical density at 436 mμ. ●, optical density at 546 mμ.

Nickerson, 1961) was employed. The cell was illuminated by unfiltered light from a Hanovia SH mercury vapor lamp at an intensity of *ca.* 10^{-9} einsteins per cm^2 per second. Periodically during the course of the reaction, filters were inserted in the light path to isolate specific lines of the lamp's output. The 436 mμ line, used to measure the thiodione concentration, was isolated by a combination of Corning 3-73 and 5-57 filters; the 546 mμ line, used to determine mercapto-menadione, was isolated by a combination of Corning 3-73 and Klett 54 filters. Optical densities at the above two wavelengths and the potential (measured against a saturated calomel electrode and reported relative to the normal hydrogen electrode) were determined every few minutes over a period of one hour. All thiodione solutions were prepared in 0.05 M phosphate buffer at *p*H 7.0, and flushed with purified nitrogen for 15 minutes prior to illumination.

Spectra.—Spectral changes in solutions that had been flushed with nitrogen were studied in an evacuable 1-cm quartz cuvet (Nickerson *et al.*, 1963). Beckman DU and Cary Model 14 spectrophotometers were used.

Paper Chromatography.—Whatman No. 1 and an isopropanol–formic acid–water (70:20:10) eluent were used in a descending system. Spots were identified by comparison with reference substances, by inspection under ultraviolet (360 mμ) light, and by reaction with ninhydrin.

Formation of Reduced Quinones.—Hydrogen over palladium, or $TiCl_3$, or $Na_2S_2O_4$ were employed to form the dihydro-derivatives of thiodione (in aqueous solution) and of mercapto-menadione (in 70% ethanol).

Detection of Sulfhydryl Compounds.—Sulfhydryl compounds formed during photochemical and hydrolytic decomposition of thiodione were detected by nitroprusside (Zimmet, 1933).

RESULTS

Spectral Changes.—A 10^{-4} M solution of thiodione in water that had been flushed with purified nitrogen for 15 minutes was illuminated by an incandescent lamp. The color of the solution changed from yellow to burgundy within a few minutes. The spectrum obtained after 5 minutes' illumination revealed a new absorption peak at 515 mμ. In the dark, in both the presence and absence of air, the burgundy color disappeared within a few hours. Treatment of an illuminated thiodione solution with a chemical reductant, such as hydrogen over palladium, or titanous chloride, decolorized the solution.

Potential Changes.—Changes in potential and optical density at 436 and 546 mμ during illumination of a 5×10^{-5} M thiodione solution by a mercury vapor lamp are shown in Figure 1. A decrease in thiodione was accompanied by an increase in the burgundy-colored photodecomposition product, and by a decrease in potential. At the end of 30 minutes the potential had fallen to −0.036 volt and had become steady, while the thiodione concentration had declined to approximately 50% of its original value. (On prolonged illumination the solution became turbid and ultimately a precipitate appeared, while the potential remained constant.) At 50% decomposition the potential attained was reasonably close to the E_0' value of thiodione (−0.025 volt at *p*H 7.0), as determined by potentiometric titration (Nickerson *et al.*, 1960). Upon subsequent aeration, the potential returned to its original value of +0.350 volt within one minute; the optical density at 546 mμ (a measure of the photodecomposition product) however remained almost unchanged for a considerable time.

These data show that photochemical formation of the burgundy product is accompanied by formation of a reduced quinone. The latter is rapidly oxidized by air, whereas the burgundy compound is stable for several hours. No burgundy intermediate was detected during reductive titration of thiodione with dithionite in neutral or alkaline solutions (Nickerson *et al.*, 1963). Thus, the burgundy photodecomposition product is not a semiquinone, and the observed drop in potential must be ascribed to the formation of dihydro-thiodione. The close agreement between the potential attained at 50% decomposition and the potentiometric E_0' value indicates that approximately equimolar amounts of thiodione and its dihydro-derivative were present when one half of the total thiodione had been decomposed. Therefore, each mole of thiodione decomposed produces one mole of dihydro-thiodione.

Identification of Photodecomposition Products.—A sulfhydryl compound was formed during photodecomposition of thiodione. The sulfhydryl compound could be extracted by chloroform or ether from an acidified solution of illuminated thiodione, and was identified as 2-methyl-3-mercapto-1,4-naphthoquinone (referred to as mercapto-menadione). Identification was established by comparison of the isolated photo-product with a synthetic reference compound. Both unknown and reference compound had identical spectra in aqueous alkali (Fig. 2) and gave identical R_F values of 0.85 in isopropanol–formic acid–water.

To determine whether mercapto-menadione was formed during illumination prior to acidification and solvent extraction, the spectrum of a 1×10^{-4} M solution of thiodione in water was examined after illumination for 2 minutes in the absence of air. The concentrations of thiodione and mercapto-menadione were calculated from their molar extinction coefficients at 410 and 515 mμ and from the observed optical densities at these wavelengths. The spectrum (Fig. 3), when corrected for the presence of 0.61×10^{-4} M thiodione, was in close agreement with that of a 0.24×10^{-4} M solution of synthetically prepared mercapto-menadione. The latter, therefore, may be presumed to be formed photochemically.

Treatment of thiodione with aqueous alkali in the

TABLE I

STOICHIOMETRY OF PHOTODECOMPOSITION OF THIODIONE (TD) TO MERCAPTOQUINONE (QSH) AND DIHYDRO-THIODIONE (TDH_2)

Original solutions, 2.0 × 10^{-4} M thiodione in water. Concentrations of TD and QSH calculated from observed optical densities at 410 and 515 mμ and by the use of the following molar absorption coefficients: TD: ϵ_{410} = 1600; ϵ_{515} = 275; QSH: ϵ_{410} = 725; ϵ_{515} = 3210.

Time of Illumination (sec.)	Conc. × 10^4 after illum.			Conc. × 10^4 after aeration		
	[TD]	[QSH]	$[TDH_2]^a$	[TD]	[QSH]	$[TDH_2]^a$
15	1.84	0.09	0.07	1.93	0.09	0
30	1.72	0.16	0.12	1.87	0.16	0
60	1.49	0.26	0.25	1.76	0.24	0
120	1.08	0.43	0.49	1.57	0.43	0

[a] Concentration of TDH_2 after illumination calculated by difference between original [TD] and sum of [TD] + [QSH]

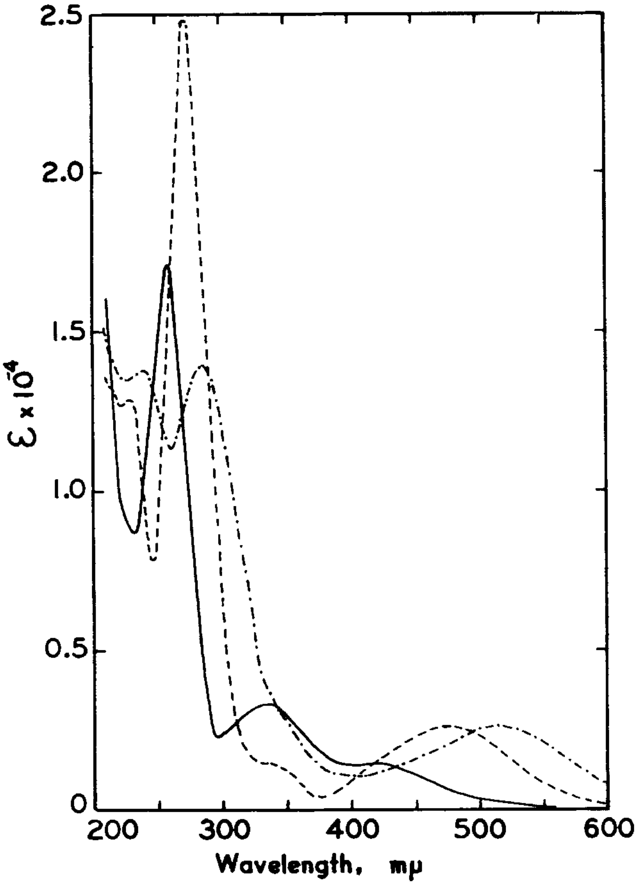


FIG. 2.—Molar absorption spectra of thiodione in water (———), of mercapto-menadione in 0.1 N NaOH (—·—·—), and of phthiocol in 0.1 N NaOH (— — — —).

dark (1 M NaOH for several hours at room temperature or a few minutes at 100°) also resulted in formation of a sulfhydryl compound. In contrast with the photodecomposition product, this sulfhydryl compound could not be extracted by chloroform. The reaction products were identified as phthiocol and glutathione by methods analogous to those given above. Phthiocol could be distinguished from mercapto-menadione by its R_F value of 0.90 (*vs.* 0.85 for mercapto-menadione) and by its spectrum (Fig. 2).

Stoichiometry of Photodecomposition.—An aqueous solution of thiodione (2.0 × 10^{-4} M) was flushed with nitrogen and illuminated for periods ranging from 15 to 120 seconds, then flushed with air for 1 minute. Optical densities at 410 and 515 mμ were read before illumination, after illumination, and after aeration. The concentrations of thiodione and mercapto-menadione were calculated from their molar extinction coefficients and the observed optical densities (Table I). The concentration of dihydro-thiodione after illumination, but before aeration, was calculated by difference (as indicated in Table I). The results show that mercapto-menadione and dihydro-thiodione were formed in approximately equimolar amounts. The rate of formation of mercapto-menadione was the same in the presence and absence of air.

Since no oxidized glutathione or other disulfide was formed, the hydrogens added to the sulfur and the quinone oxygens did not arise as a result of oxidation of a sulfhydryl compound.

DISCUSSION

Thiodione (2-methyl-3-S-glutathionyl-1,4-naphthoquinone) can be decomposed either photochemically or hydrolytically. The former reaction is unaffected by *p*H; the latter, a hydrolytic cleavage of the sulfur-ring carbon bond, is catalyzed by alkali and is not influenced by light. The completely different conditions for cleavage of one or the other of the bonds between sulfur and its neighboring carbons indicate that the hydrolysis reaction is ionic, whereas photochemical cleavage probably proceeds by a homolytic mechanism.

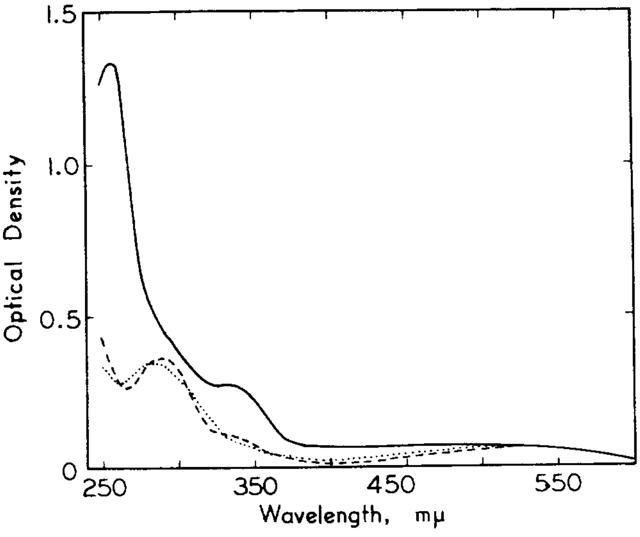


FIG. 3.—Evidence of formation of mercapto-menadione during illumination of thiodione in aqueous solution. ———, spectrum obtained after illumination of 1 × 10^{-4} M thiodione for 2 minutes; — — —, difference spectrum, due to mercapto-menadione formed photochemically (= observed spectrum minus spectrum of 0.61 × 10^{-4} M thiodione); ·····, spectrum of synthetic mercapto-menadione (0.24 × 10^{-4} M). Calculations for concentrations of thiodione and of mercapto-menadione in the illuminated solution are subject to an error of ± 5%.

Photochemical reduction of quinones by ultraviolet light in the presence of substances able to donate hydrogen, *e.g.*, ethanol, is known (Ciamician and Silber, 1901; Berthoud and Porret, 1934; Paoloni and Marini-Bettolo, 1957). The primary step, according to Bridge and Porter (1958), is an abstraction of hydrogen from the donor, giving a semiquinone radical (equation 1), or, in alkaline solution, a semiquinone radical-ion (equation 2). Alternatively, photo-

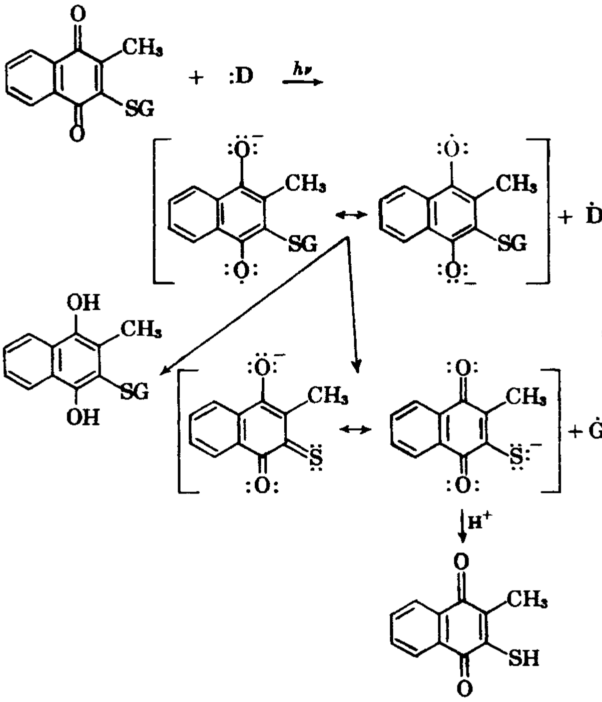
$$Q + RH \xrightarrow{h\nu} \dot{Q}H + \dot{R} \qquad (1)$$

$$\dot{Q}H \longrightarrow H^+ + \dot{Q}^- \qquad (2)$$

reduction of quinones may occur by a charge transfer process (equation 3), which in this particular case involves a σ-electron donor and a π-electron acceptor (Mulliken, 1952; Kosower, 1960). Dissociation of the

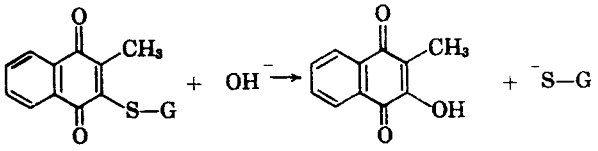
$$Q + :D \rightleftharpoons [Q, :D] \xrightarrow{h\nu} [\dot{Q}^- \dot{D}^+] \longrightarrow \dot{Q}^- + \dot{D}^+ \qquad (3)$$

charge transfer complex (shown as the last step in equation 3) is not required for reduction of the quinone, if "reduction" is defined as conversion of a quinoid to a benzenoid structure. By analogy, a charge transfer process can account for the photodecomposition of thiodione with the formation of equimolar amounts of dihydro-thiodione and mercapto-menadione, as shown in equation (4), in which the donor (:D) may be water.



(4)

Hydrolysis of thiodione to phthiocol and glutathione appears to proceed by a nucleophilic attack by OH^- on the ring carbon (equation 5). Specific cleavage of the C—S bond proximal to, rather than distal from, the ring is favored by the greater electron deficiency of the (unsaturated) ring carbon, relative to the β-carbon of the cysteinyl residue. Alkali-catalyzed cleavage of thiodione may be contrasted with the action of alkali on vitamin K_1. In the latter case, the reaction product has a higher molecular weight than the starting material (probably owing to the addition of two oxygens; Almquist and Klose, 1939), whereas hydrolysis of thiodione yields phthiocol.



(5)

It is a remarkable fact that phthiocol, originally isolated by saponification and ether extraction of the acetone-soluble fat obtained from human tubercle bacilli (Anderson and Newman, 1933), can also be obtained by alkaline hydrolysis of thiodione, but not from vitamin K_1. Phthiocol does not appear to occur free in tubercle bacilli (Parshin, 1946; Francis *et al.*, 1949). The present findings suggest that a 2-methylnaphthoquinone bound *via* a thioether linkage may be the precursor of phthiocol. Curiously, the presence of sulfur in the lipid fraction of tubercle bacilli has not been suspected. Thus, bound forms of thiodione may occur in nature.

REFERENCES

Almquist, H. J. (1936), *J. Biol. Chem. 114*, 241.

Almquist, H. J. (1937), *J. Biol. Chem. 117*, 517.

Almquist, H. J., and Klose, A. A. (1939), *J. Biol. Chem. 130*, 791.

Anderson, R. J., and Newman, M. S. (1933), *J. Biol. Chem. 101*, 773.

Bridge, N. K., and Porter, G. (1958), *Proc. Roy. Soc. (London) Ser. A 244*, 259, 276.

Berthoud, A., and Porret, D. (1934), *Helv. Chim. Acta 17*, 694.

Ciamician, G., and Silber, P. (1901), *Ber. 34*, 1530.

Ewing, D. T., Tomkins, F. S., and Kamm, O. (1943), *J. Biol. Chem. 147*, 233.

Francis, J., Madinaveitia, H. M., Nacturk, H. M., and Snow, G. A. (1949), *Nature 163*, 365.

Kosower, E. M. (1960), *in* The Enzymes, ed. 2, Vol. 3, Boyer, P. D., Lardy, H., and Myrbäck, K., editors, New York, Academic Press, Inc., Chap. 13.

MacCorquodale, D. W., Brinkley, S. B., McKee, R. W., Thayer, S. A., and Doisy, S. E. (1939), *Proc. Soc. Exp. Biol. Med. 40*, 482.

Mulliken, R. S. (1952), *J. Am. Chem. Soc. 74*, 811.

Nickerson, W. J., Falcone, G., and Strauss, G. (1960), *Fed. Proc. 19*, 35.

Nickerson, W. J., Falcone, G., and Strauss, G. (1963), *Biochemistry 2*, 537 (this issue).

Paolini, L., and Marini-Bettolo, G. B. (1957), *Gazz. Chim. Ital. 87*, 395.

Parshin, A. N. (1946), *Biokhimiya 11*, 53.

Strauss, G., and Nickerson, W. J. (1961), *J. Am. Chem. Soc. 83*, 3187.

Zimmet, D. (1933), *Compt. rend. Soc. Biol. 113*, 984.