On the basis of these results duplicate 100ml. cultures of medium were prepared, each incorporating 100mg. of [$Me-^2H_3$]methionine instead of unlabelled methionine. The cultures were inoculated and grown as described above. The isolated poriferasterol was checked by gas-liquid chromatography (98% pure) and then analysed by mass spectrometry (Fig. 1). A large peak at m/e 412 was noted and represents the molecular ion peak for the non-deuterated poriferasterol present. Large peaks were also observed at m/e 413, 415 and 416, and were due to poriferasterol containing 1, 3 and 4 deuterium atoms/mol. respectively. The molecular ions at m/e 414 and 417 were of the order expected for the ^{13}C isotope content of the peaks at m/e 413 and 416 respectively. The ions at m/e 397, 379, 369 and 351, corresponding to the loss of methyl, methyl plus water, terminal isopropyl and terminal isopropyl plus water respectively were all accompanied by ions at m/e values +1, +3 and +4. The peak at m/e 300 for loss of part of the side chain (Lenfant *et al.* 1967) was unaccompanied by higher m/e peaks, thus providing evidence that all four deuterium atoms were present in the sterol side chain. The peaks at m/e 271 and 255 also supported this conclusion.

This result establishes that in *O. malhamensis* alkylation of the sterol side chain proceeds via a mechanism leading to the intermediate formation of a 24-ethylidene steroid (III), which is subsequently reduced to give the 24-ethyl derivative (VII). This is in agreement with our previous results, which also indicated that during β -sitosterol synthesis in higher plants a 24-ethylidene steroid is an intermediate (Goad & Goodwin, 1966, 1967; Goad *et al.* 1966). However, Lenfant *et al.* (1967) have established that in the slime mould *D. discoideum* an alternative mechanism for the stabilization of the carbonium ion (II) is in operation (e.g. see Scheme 1). The recent isolation from plant sources

of clerosterol (Sucrow, 1966; Manzoor-i-Khuda, 1966), which has side chain (IV), also supports the conclusion that an alternative mechanism can occur as indicated in Scheme 1. In this Scheme the problem of the formation of the 22,23-double bond frequently encountered in phytosterols is not considered.

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Reduction of a Disulphide Bond of Ribonucleotide Reductase by the Dithiol Substrate

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A ribonucleotide reductase from *Lactobacillus leichmannii* catalyses the reduction of ribonucleoside triphosphates to the corresponding deoxyribonucleotides by dithiols, and has an absolute

requirement for deoxyadenosylcobalamin (Blakley, 1966). Dihydrolipoic acid and other 1,3- or 1,4-dithiols are suitable reductants at high concentrations (30mM), whereas monothiols such as 2-

mercaptoethanol are relatively ineffective. However, the physiological reductant is thioredoxin, a low-molecular-weight protein with a single disulphide bridge that is reduced by an NADPH-linked flavoprotein, thioredoxin reductase. Ribonucleotide reductase also catalyses the exchange of hydrogen between water and the cobalt-bound methylene group of deoxyadenosylcobalamin, and this reaction is believed to be an integral part of the sequence of reactions involved in ribonucleotide reduction (Hogenkamp, Ghambeer, Brownson & Blakley, 1967).

Preparations of the ribonucleotide reductase currently in use appear homogeneous on ultracentrifugation (mol.wt. about 70000); however, on electrophoresis in polyacrylamide gel they behave as though 50–70% pure. Such preparations have been used in substrate amounts to investigate interaction of the enzyme with the dithiol substrate. When thioredoxin, thioredoxin reductase and NADPH were used as the reducing system, spectrophotometric measurements showed that 1 mole of NADPH was oxidized/70000g. of protein in the enzyme preparation. No oxidation of NADPH occurred if any of the components was omitted from the reaction mixture. This result suggests that one disulphide bridge on the ribonucleotide reductase is reduced by the thioredoxin system. After interaction of other thiols with ribonucleotide reductase and the removal of excess of thiol by dialysis, the extent to which they had reduced the enzyme could be determined by measuring how much additional reduction occurred in the presence of the thioredoxin system. Dihydropyridine (30mm) was almost as effective a reductant as thioredoxin, but mercaptoethanol was relatively ineffective even at higher concentration (0.1M).

Ribonucleotide reductase used in these studies was only slightly inactivated by iodoacetamide (20–50-fold molar excess) at pH 7.5 and 0°. The inactivation varied from 0 to 40% in various preparations. After reduction, however, the reductase was rapidly inactivated under these conditions, the extent of inactivation being about 95% after 15 min. Amino acid analysis indicated that the inactivated enzyme contained about 2.4 residues of *S*-carboxymethylcysteine/mol. of mol.wt. 64 000. No other modified amino acid residues could be found. These results clearly indicate the reduction of a protein disulphide bridge by the dithiol substrate. The appearance of free dithiol groups on the enzyme was also indicated by reaction of the reduced reductase with 5,5'-dithiobis-(2-nitrobenzoic acid).

Iodoacetate reacts with the reduced enzyme in a similar manner to iodoacetamide. The reduced enzyme was treated with [1-¹⁴C]iodoacetate and subjected to tryptic digestion, and the resultant

peptides were separated by paper electrophoresis at pH 4.7 in one dimension and by chromatography in butan-1-ol-acetic acid-water (4:1:5, by vol.) in the second dimension (Katz, Dreyer & Anfinsen, 1959). After radioautography only one or possibly two radioactive peptides could be detected, a result suggesting that only one disulphide bond is opened by reduction.

The reduced enzyme is reoxidized in the presence of deoxyadenosylcobalamin and a ribonucleotide substrate such as GTP. If, after reduction of ribonucleotide reductase by the thioredoxin system, GTP and deoxyadenosylcobalamin were added, GTP reduction proceeded rapidly until all the NADPH was exhausted. Deoxyadenosylcobalamin was then decomposed by photolysis to prevent further GTP reduction on the subsequent addition of NADPH. However, oxidation of added NADPH did occur to an extent equal to that which was observed when the thioredoxin system was first added to the enzyme. This experiment demonstrates that on incubation with GTP and deoxyadenosylcobalamin the ribonucleotide reductase is again largely converted into the disulphide or oxidized form. To obtain further evidence on this point, the ribonucleotide reductase was first reduced by the thioredoxin system with only a slight excess of NADPH. During further incubation of the reaction mixture this excess of NADPH was entirely removed by an NADPH oxidase present as a contaminant in the enzyme preparation. After removal of NADPH in this way, GTP and deoxyadenosylcobalamin were added and incubation was continued. Subsequent analyses showed that under these conditions, in which reduced enzyme is the only reductant, deoxyGTP was formed in an amount equivalent to 70–80% of the enzyme disulphide bonds previously reduced by NADPH. Simultaneously, the protein thiol groups alkylated by iodoacetamide decreased to 25% of the value for the reduced enzyme.

These results clearly establish that a disulphide bridge on ribonucleotide reductase is reduced in the presence of the dithiol substrate, and is reoxidized by the ribonucleotide substrate in the presence of deoxyadenosylcobalamin. Other evidence appears to indicate that reduction of the disulphide link is essential for activity; however, the enzyme probably does not alternate between oxidized and reduced forms during reduction of ribonucleotides. A mechanism in which the enzyme is first reduced by reduced thioredoxin with liberation of oxidized thioredoxin from the enzyme, and the reduced enzyme then reoxidized directly by deoxyadenosylcobalamin and ribonucleotide with liberation of deoxyribonucleotide, would constitute a Ping Pong mechanism in the nomenclature of Cleland (1963). Such reactions are characterized by a family of

parallel plots of reciprocal velocity against reciprocal concentration of the variable substrate when different fixed concentrations of the second substrate are used. In the case in question, linear double-reciprocal plots were obtained with thioredoxin as variable substrate, but plots at different fixed concentrations of GTP showed a small but definite difference in slope. This result indicates that cyclic reduction and oxidation of the enzyme is not kinetically detectable. Evidence obtained from studies on hydrogen transfer from 5'-deoxy-[5'-³H]adenosylcobalamin to water also appears to be inconsistent with direct reduction of ribonucleotide by the enzyme. We consider this hydrogen transfer to be due to the first of the sequence of reactions involved in ribonucleotide reduction (Hogenkamp *et al.* 1967), in which the cobamide is reduced by the dithiol to a transitory intermediate, which then donates a hydride ion to the 2'-carbon of the nucleotide. If the dithiol in fact reduces the protein disulphide bridge and the pair of protein thiol groups thus formed are the obligatory reductant of the nucleotide, then the protein thiol groups should be capable of reducing deoxyadenosylcobalamin to the transitory intermediate and therefore of catalysing hydrogen transfer from deoxyadenosylcobalamin to water. In fact enzyme reduced by dihydrolipoate (which was then removed), though fully active for catalysing such hydrogen transfer in the presence of dihydrolipoate or reduced thioredoxin, showed little activity for catalysing the transfer in absence of these dithiols.

These results are consistent with the view that the reduction of GTP to deoxyGTP by the reduced ribonucleotide reductase is mediated by the catalytic amount of thioredoxin present in the reaction mixture. It is assumed that reduction of the ribonucleotide reductase by reduced thioredoxin is freely reversible, so that the following reactions occur:

This cycle can continue as long as reduced reductase is available. Evidence to support this concept was found in the observation that addition of a catalytic amount of thioredoxin (oxidized) to the reduced enzyme significantly enhanced the rate of hydrogen transfer between deoxyadenosylcobalamin and water. A large excess of thioredoxin was inhibitory, however, presumably because too little reduced enzyme now remained to catalyse the second of the above reactions. Direct evidence of the first of the above reactions was obtained by reducing reductase with dihydrolipoate, removing dihydrolipoate by dialysis and allowing the reduced enzyme to react with thioredoxin. On pressure dialysis of the mixture about 30% of the thioredoxin passed through the dialysis bag and was shown to be in the reduced form by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). No thiol was detected in a similar diffusate when thioredoxin was omitted from the mixture. A corresponding loss of thiol groups from the protein remaining in the dialysis bag could be demonstrated in the case where thioredoxin was added.

In summary, the evidence presented indicates that the dithiol substrate reduces a disulphide bridge on the ribonucleotide reductase, converting the enzyme into its active reduced form. When thioredoxin is the reductant the reduction is freely reversible, so that enzyme thiol groups can act as the ultimate reductant for ribonucleotide.

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