ΔH_B^{eff}, the values derived for E_a, particularly above the break, were sharply reduced from the true values. In the particular case shown, the E_a value that would be determined above the break is only 5.3 kcal mole^{-1} when ΔH_B^{eff} is -15 kcal mole^{-1}, while the true E_a value in this temperature region is 15 kcal mole^{-1}. Another frequently observed result was that the break was shifted to appreciably lower temperatures than the true break (in E_a for V_{max}) when somewhat lower substrate concentrations (two to ten times the K_m at 5°C) were employed.

In a second set of simulations, we assumed E_a to be constant and ΔH_B^{eff} to be finite and negative over the temperature range of interest. In such cases, a wide range of combinations of E_a, ΔH_B^{eff}, and substrate concentration yield strongly curved Arrhenius plots; when there is any significant amount of experimental scatter of the data points, such curves can convincingly mimic biphasic, linear Arrhenius plots. One example of such behavior is shown in Fig. 1b, where values for E_a and ΔH_B^{eff} comparable to those determined for the adenosinetriphosphatase in oleate-enriched membranes were employed.

These simulations indicate that a variety of errors, most notably artifactual breaks, can arise in Arrhenius plots when temperature-dependent increases of K_m are not accounted for; similar results are found if K_m decreases with increasing temperature, except that E_a values tend to be over- rather than underestimated in this case. To avoid such errors, all of the relevant kinetic parameters of the membrane-bound enzyme under investigation should be determined for at least three temperatures which encompass the entire temperature range to be studied. If it proves impossible to derive a single set of optimal conditions for all temperatures, V_{max} should be determined by means of Eadie-Hofstee or Lineweaver-Burk plots at all temperatures studied, with careful attention to other parameters such as ion affinity constants or pH variations with temperature. While a few reports of a temperature dependence of K_m or of a substantial enthalpy of substrate binding to membrane enzymes have appeared (*9*), the magnitude and significance of this effect do not seem to be generally appreciated in the existing literature, where the temperature dependence of enzyme kinetic parameters other than V_{max} is frequently neglected when Arrhenius plots for membrane enzymes are interpreted.

The results reported here clearly demonstrate that serious errors in the interpretation of Arrhenius plots can arise if temperature variations in substrate-binding affinity are not taken into account when determining the effect of temperature on the rate of the catalyzed reaction. As the relationship of membrane fluidity to the activity of membrane enzymes is at present an area of considerable interest and controversy, such errors in interpretation may seriously hamper subsequent attempts to develop realistic models of the nature of enzyme-membrane interactions.

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10. We thank N. Bell and D. P. Sharma for their technical assistance. This work was supported by the Alberta Heart Foundation. J.R.S. is the recipient of a studentship award from the Medical Research Council of Canada.

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Sulfhydryl Groups and the Monodeiodination of Thyroxine to Triiodothyronine

Abstract. *Sulfhydryl reagents exert a profound influence on the monodeiodination of thyroxine to triiodothyronine by rat and sheep tissues in vitro. A marked dithiothreitol-induced increase in the monodeiodination by fetal sheep liver homogenates suggests that the characteristically low conversion in fetal tissues is related more to the status of sulfhydryl groups than to a deficiency of the monodeiodinating enzyme.*

3,5,3′-Triiodothyronine (T_3) is characteristically present at a much lower concentration in the circulation of the fetus than in the adult. Immediately after birth, however, there is a marked increase in serum T_3 (*1*). This increase in the availability of T_3, a very potent thyromimetic agent, may play a vital role in maintaining the body temperature of the newborn when the warm intrauterine environment is no longer available. The lower serum T_3 concentration in the fetus than in the adult is due to a lower rate of conversion of thyroxine (T_4) to T_3 in extrathyroidal tissues (*2*). Recent studies indicate that this process of outer-ring monodeiodination of T_4 to T_3 is enzymatic in nature (*3*). The extrathyroidal tissues of the fetus, such as liver, have also been shown to convert T_4 to T_3 in vitro to a much lesser extent than the tissues of the adult or the newborn; kinetic studies of the conversion with liver tissue indicate significant differences in the maximum rate of the reaction, V_{max}, as defined by the Michaelis equation, and not in the Michaelis constant, K_m (*4*). However, it is not known whether reduced conversion of T_4 to T_3 in the fetus is related primarily to a deficiency in the amount of the putative iodothyronine monodeiodinase or to inactivation of the existing enzyme—for example, through a conformational change in the enzyme which may not permit appropriate activity. The study reported here demonstrates that sulfhydryl groups of tissues are essential to their ability to monodeiodinate T_4 to T_3. The data also suggest that T_4 outer-ring monodeiodinase is plentiful in the tissues of the fetus but is

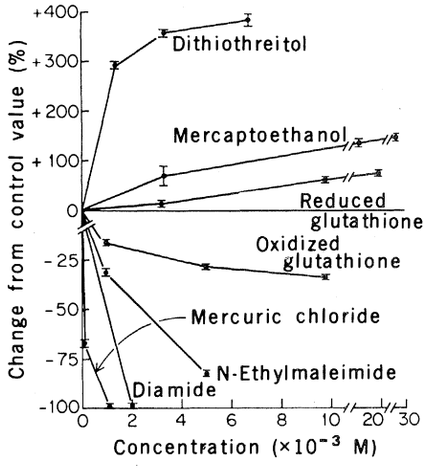

Fig. 1. Effect of various sulfhydryl reagents on monodeiodination of T_4 to T_3 by rat liver homogenate. The T_3 produced in the absence of a sulfhydryl agent (control) was arbitrarily assigned a value of 100, and the product in the presence of a sulfhydryl reagent was expressed as a percentage of the control value; each result is the mean ± standard error (S.E.) for triplicate or quadruplicate determinations. *N*-Ethylmaleimide and mercuric chloride were obtained from Baker Chemical Company. All other reagents were obtained from Sigma Chemical Company.

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inactive, probably because of the relative unavailability of sulfhydryl groups of the tissues.

The conversion of T_4 to T_3 was studied by incubating approximately 130-mg (wet weight equivalent) portions of freshly prepared liver homogenates in 0.15*M* phosphate buffer (*p*H 7.3) with 2 μg of T_4 for 15 minutes at 37°C and quantifying the amount of T_3 generated by radioimmunoassay (RIA) of ethanol extracts of the incubation mixture (*5*). The amount of T_3 produced in 26 experiments with six different pools of rat liver homogenates was 115 ± 9 ng per microgram of T_4 per gram equivalent of tissue per hour [mean ± standard error (S.E.)].

The effect of agents that may influence tissue thiol groups on the monodeiodination of T_4 to T_3 is shown in Fig. 1. Various thiol-protective agents, including dithiothreitol (DTT), mercaptoethanol, and reduced glutathione, stimulated the monodeiodination. The sulfhydryl-oxidizing agent diamide [azodicarboxylic acid bis(dimethylamide)], oxidized glutathione, and the sulfhydryl-binding agents *N*-ethylmaleimide and mercuric chloride markedly reduced the monodeiodination. These results strongly suggest that sulfhydryl groups play an important role in the monodeiodination of T_4 to T_3 by liver homogenate.

Rat tissues other than liver and kidney are known to be, on a per gram basis, very weak in the conversion of T_4 to T_3 (*5*). Dithiothreitol could be shown to cause some increase in the converting activity of most of the tissues so studied (*6*). However, the converting activity in tissues other than liver and kidney was much less than that in liver and kidney even in the presence of DTT (*6*). These findings indicate that the low T_4-monodeiodinating activity of tissues other than liver and kidney is the result of a relative deficiency in the amount of putative T_4 outer-ring monodeiodinase and probably not of a difference in the relative enzyme activity secondary to differences in the available sulfhydryl groups.

To gain insight into the mechanism of the low conversion of T_4 to T_3 by fetal tissues, studies similar to those described above were conducted with liver homogenates of maternal and fetal sheep at 135 days gestation. As noted elsewhere (*4*), fetal liver generated much less T_3 from T_4 than maternal liver (Fig. 2). However, when DTT was present in the incubation medium, the amount of T_3 produced by fetal liver became at least as great as that produced by maternal liver. This effect of DTT on fetal liver was strikingly different from that on adult tissues with low converting activity described above. It suggests that the poor ability of fetal tissues to monodeiodinate T_4 to T_3 is not due to a quantitative deficiency in the amount of the outer-ring monodeiodinase but rather to inactivation of existing enzyme, possibly through a high state of oxidation of protein sulfhydryl groups.

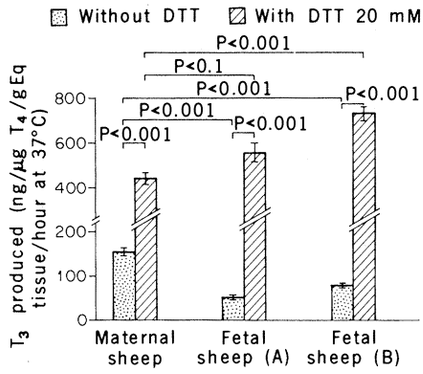

Fig. 2. Effect of dithiothreitol (*DTT*) on the conversion of T_4 to T_3 by liver homogenates of a maternal sheep and two sheep fetuses (gestational age, 135 days); the data are expressed as mean ± S.E. for quadruplicate determinations. The amount of T_3 produced from T_4 by fetal tissues in the presence of DTT was at least as great as that produced by maternal tissues. Similar results were obtained with two other combinations of maternal and fetal sheep.

The effect of DTT on the kinetics of conversion of T_4 to T_3 by liver homogenates of fetal sheep was also examined (*7*). Dithiothreitol caused a marked increase in V_{max} in these studies, but there was little or no change in K_m (*7*). These changes were very similar to those observed previously in the kinetics of conversion of T_4 to T_3 in liver homogenates of newborn sheep compared to those of fetal sheep (*4*).

The effects of sulfhydryl reagents on the conversion of T_4 to T_3 by peripheral tissues suggest that outer-ring monodeiodination of T_4 is closely related to the status of sulfhydryl groups in the tissues. Sulfhydryl groups may influence monodeiodination by one or both of the following two mechanisms: (i) protein-bound sulfhydryls may influence enzyme conformation and (ii) a free sulfhydryl cofactor, such as glutathione, may act as a coenzyme. A recent study has suggested the existence of substances in peripheral tissues which mimic the effects of DTT (*8*). In addition, the concentration of nonprotein sulfhydryl groups in the liver has been found to be significantly lower in fetal sheep than in neonatal and adult sheep (*9*). These results support the hypothesis that changes in the tissue concentration of sulfhydryl groups play an important role in the sudden changes in the monodeiodination of T_4 to T_3 observed during the transition from intrauterine to extrauterine life.

In addition to a lower serum T_3 concentration, the fetus characteristically has a much higher serum reverse T_3 (3,3′,5′-T_3, rT_3) concentration than the adult. Kinetic studies indicate that this is due to both reduced clearance and increased production of rT_3 (*2*). However, increased fetal rT_3 production appears to be related mainly to increased availability of its precursor, T_4. Since the ratios of daily production rates of rT_3 and T_4 are similar in the fetus and the adult, the actual rate of conversion of T_4 to rT_3 in the fetus is probably not much different from that in the adult (*2*). This information and the data reported here raise interesting questions concerning the relative effects of tissue sulfhydryl groups on the conversion of T_4 to rT_3 and to T_3. Information related to this is not yet available. Some data have been obtained, however, on the role of sulfhydryl groups in the degradation of rT_3 to 3,3′-diiodothyronine (T_2). Preliminary studies suggest that, as in the conversion of T_4 to T_3, sulfhydryl groups are important in the degradation of rT_3 to T_2. These findings may explain the combination of diminished T_3 production and diminished rT_3 clearance in the fetus.

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5. Since it takes 30 minutes or more for the T_4 monodeiodination reaction to reach equilibrium, the 15-minute incubation period reflects the initial rate of reaction. Incubation mixtures were extracted with two volumes of absolute ethanol and T_3 was measured in the extracts as described previously [I. J. Chopra, *Endocrinology* **101**, 453 (1977)].
6. The amounts of T_3 produced during incubation of homogenates of rat liver, kidney, muscle, spleen, and brain were (means of quadruplicate determinations, nanograms per microgram of T_4 per gram equivalent of tissue per hour) 122, 120, 19.0, 10.8, and 17.2, respectively, in the absence

of DTT and 436, 490, 48.4, 34.6, and 15.5, respectively, in the presence of 20 m*M* DTT.

7. For kinetic studies, tissue homogenates were incubated with various doses of T_4 for 15 minutes at 37°C in the absence and in the presence of DTT, and the data on the amount of T_3 produced were examined by using a Lineweaver-Burk plot [H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56**, 658 (1934)]. The K_m for conversion of T_4 to T_3 by fetal liver homogenate was 5.5 μM in fetuses A and B (Fig. 2) in the absence of DTT and 5.5 and 6.25 μM, respectively, in the presence of 20 m*M* DTT. The V_{max} (nanograms of T_3 per gram equivalent of tissue per hour) was 288 in fetus A and 330 in fetus B in the absence of DTT and 2310 and 3078, respectively, in the presence of 20 m*M* DTT. The K_m for conversion of T_4 to T_3 by maternal liver homogenate (Fig. 2) was 1.5 μM in the absence and 2.5 μM in the presence of 20 m*M* DTT, whereas V_{max} (ng/g-eq hour) was 576 in the absence and 2881 in the presence of 20 m*M* DTT.
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9. Total and nonprotein sulfhydryl groups were measured by using Ellman's reagent [J. Sedlak and R. H. Lindsay, *Anal. Biochem.* **25**, 192 (1968)] in liver homogenates of four fetal sheep (gestational age, 132 to 137 days), four neonatal sheep (2 to 8 hours after delivery), and four maternal sheep. The mean content of total sulfhydryl groups in fetal liver, 16.7 ± 2.3 mmole/kg (wet weight), was significantly ($P < .05$) lower than that (23.2 ± 1.3 mmole/kg) in maternal liver but did not differ significantly from that (19.7 ± 1.8 mmole/kg) in neonatal liver. However, the mean content of nonprotein sulfhydryl groups in fetal liver (2.2 ± 0.40 mmole/kg) was significantly ($P < .005$) less than that in maternal (4.6 ± 0.38 mmole/kg) as well as neonatal (5.5 ± 0.52 mmole/kg) liver.
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Methylation of Humic Acid Fractions

Abstract. *A new three-step permethylation procedure for humic acid fractions has been developed. In the first step, carboxylic acid groups are methylated with diazomethane in dimethylformamide; in the second step, hydroxyl groups are methylated with methyl iodide and dimethyl sulfinyl carbanion in dimethylformamide; and in the third step, acidic groups hydrolyzed in the earlier steps are methylated with diazomethane. The resulting product is completely soluble in methylene chloride (a high degree of methylation has been achieved).*

Humic substances are the most abundant group of organic macromolecules in natural waters and soils. They are also among the most reactive materials in the environment, interacting strongly with practically all of the other organic and inorganic components of natural water systems (*1*).

Traditionally humic substances have been divided into three major fractions—humic acid, fulvic acid, and humin—on the basis of their differing solubilities in basic and acidic solutions. In this report we shall limit our discussion to humic acid.

In spite of their importance in environmental chemistry, very little is known about the chemical structure of the humic acids. In practically all of the studies on structural elucidation of the humic acids, degradation procedures have been used which have yielded only low-molecular-weight fragments (*2*).

Most workers who have studied the chemical structure of humic acids have used the traditional fractionation scheme without attempting to obtain further chemical fractionation of their preparations, even though it is generally recognized that they are mixtures of chemical species. Instrumental techniques such as infrared spectroscopy (IR) and nuclear magnetic resonance spectroscopy (NMR) have therefore yielded only the most general information, whereas in chemical degradation studies it has been necessary to use reagents which attack a variety of different chemical bonds. It has not been possible to use the highly specific degradation techniques which have been successful in elucidating the chemical structure of other natural products such as polysaccharides, steroids, or proteins because humic acids have proved to be highly resistant to chemical attack by many of the reagents that are used for partial degradation. In addition, unfractionated humic acids are insoluble in most organic solvents. We have suggested (*3*) that this resistance to chemical attack arises from the fact that humic acids form molecular aggregates in solution that must be disaggregated in order that specific functional groups in the humic acid molecules be exposed to chemical reaction.

Our approach to the elucidation of the chemical structure of humic acids is based upon a model we have proposed elsewhere (*3*). In this model humic acids are depicted as being made up of molecular aggregates that are held together by noncovalent and weak covalent bonds. In order to determine the chemical structure for humic acid, it is necessary to disrupt this aggregation and to isolate the various chemical species. The first step in this approach is the fractionation of natural humic acids by adsorption chromatography on Sephadex (*4, 5*). We have shown that fractionation breaks up "mixed aggregates" that are composed of several different chemical species. The next step consists of the elimination of hydrogen bonding with a concomitant reduction in aggregation. This reduction of hydrogen bonding is accomplished by replacing the protons in acidic, phenolic, and alcoholic groups with methyl groups. After this derivatization, the humic acid fractions are further fractionated and each of the fractions is isolated and examined by conventional instrumental techniques. If necessary, controlled degradation reactions are carried out on each of the fractions.

A wide variety of different methylation procedures has been tried on humic acid preparations (*6, 7*). In practically all of the procedures the humic acid is suspended in solvent, and methylation reagents such as dimethyl sulfate and potassium carbonate, methyl iodide and silver oxide, or diazomethane are added to the mixture. These methods have all of the disadvantages inherent in reactions carried out on heterogeneous mixtures. In an earlier paper we reported that humic acid fractions can be dissolved in pyrrolidone and methylated with diazomethane (*7*). Diazomethane will methylate carboxyl groups and some highly acidic phenolic groups; however, weakly acidic phenolic groups and carbohydrate alcoholic groups, which are also probably present in humic acids (*1*), will not react. Therefore, it was necessary to develop another procedure for more complete methylation.

We have found that the methyl esters of humic acid fractions that have been prepared by diazomethane methylation may be further methylated by a modification of the procedure developed by Hakomori (*8*) for the permethylation of saccharides in which methyl sulfinyl carbanion is the base and methyl iodide is the methylating reagent. The methylated humic acid fractions obtained in this way are completely soluble in both methylene chloride and chloroform.

The initial esterification step with diazomethane is necessary because without it the product obtained from the Hakomori reaction is not soluble in methylene chloride or in chloroform. The fact that the methylated products are completely soluble in these solvents indicates that a high degree of methylation has been achieved; NMR and IR studies have provided additional evidence of this methylation.

The permethylation procedure that we have developed is simple and straightforward. The humic acid fractions were isolated as outlined by Wershaw *et al.* (*7*); dissolved oxygen was purged from the solutions with nitrogen during all the steps in which the *p*H was above 8, including the initial extraction of humic

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