Sulphite-ion-catalysed Reversion of 5,6-Dihydrothymine-6-sulphonate to Thymine. A General-base-catalysed Reaction

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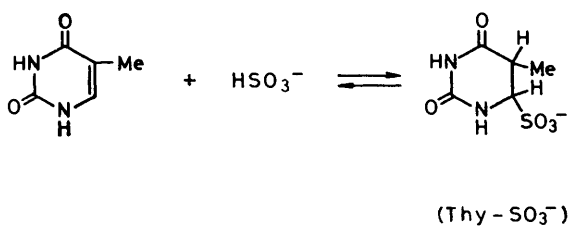
5,6-Dihydrothymine-6-sulphonate (Thy-SO_3^-) was isolated as its sodium salt from a reaction mixture of thymine and sodium hydrogensulphite. Thy-SO_3^- was stable in unbuffered aqueous solution, but reverted to thymine in the presence of general bases; *e.g.*, sulphite (SO_3^{2-}) and phosphate (HPO_4^{2-}) ions were efficient catalysts for this reversion in neutral solutions. The rate of the reversion was first-order in base, and the rate of addition of hydrogensulphite to thymine was proportional to the square of the hydrogensulphite concentration. The pH-independent equilibrium constant of the reaction, thymine + HSO_3^- $\rightleftharpoons$ Thy-SO_3^-, was determined, and it was in good agreement with the expected equilibrium constant that was calculated from the observed rates of the forward and the reverse reactions. The rate of reversion of 5-[^{2}H]Thy-SO_3^- to thymine in phosphate buffers was 4.11-fold lower than that of Thy-SO_3^-, indicating that the proton transfer at position 5 is the rate-determining step in the reversible reaction. Mechanisms that are consistent with the observed kinetics are discussed.

THE addition of hydrogensulphite to pyrimidine nucleosides to form 5,6-dihydropyrimidine-6-sulphonate derivatives has been widely used in the chemical modification of nucleic acids.[1] The mechanism of the reversible addition of hydrogensulphite to 2,4-dioxopyrimidines has been a current subject of research in several laboratories.[2-4] Whereas the addition to uracil was analysed in detail, the addition to thymine was only briefly described in earlier reports [5,6] and was recently investigated to some depth by Shapiro and his co-workers.[4] An aspect of the reversion of 5,6-dihydrothymine-6-sulphonate (Thy-SO_3^-) to thymine was previously investigated by us during a study of the hydrogensulphite-mediated deamination of 5-methylcytosine.[7] In these studies, however, the product of the reaction, Thy-SO_3^-, was not isolated, and therefore it is not known what factors determine the rate of the reversion. Specifically, one can ask the question: what causes the reversion of Thy-SO_3^- to thymine in the equilibrium between thymine and Thy-SO_3^- in hydrogensulphite solution, the hydroxide ion, the sulphite ion, or the hydrogensulphite ion?

We have now isolated Thy-SO_3^- as its sodium salt and have studied the reversion of Thy-SO_3^- to thymine in detail. We now report the results of these studies which provide a reasonable explanation for the equilibrium between thymine and Thy-SO_3^- in the presence of hydrogensulphite (Scheme 1).



SCHEME 1

RESULTS

Isolation and Properties of Thy-SO_3^-.—Isolation of Thy-SO_3^- from the reaction mixture containing thymine and a large excess of sodium hydrogensulphite was achieved by fractional precipitation with ethanol. The yield and purity of the crude products obtained in repeated experiments showed considerable variance as might be expected from such a procedure. However, this simple procedure was very effective in obtaining the sodium salt of Thy-SO_3^- free from thymine, and the quality of the material was satisfactory for use in the present study.

Isolation of Thy-SO_3^- could also be effected by paper chromatography using ethanol–water (7 : 3 v/v) as the developing solvent. In this procedure too, desalting required repeated precipitation of the isolated material. We have also observed that 5,6-dihydrothymidine-6-sulphonate can be isolated by this chromatographic technique from reaction mixtures of thymidine and sodium hydrogensulphite (R_F 0.44, as compared to R_F 0.65 for thymidine).

As expected, the Thy-SO_3^- sodium salt showed an end absorption in its u.v. spectrum, and behaved as an anion in electrophoresis. The n.m.r. signals observed for this material in ^2H_2O were the same as those previously reported for this compound [7] and indicated that the addition of a proton and SO_3^{2-} across the 5,6-double bond was *trans*. Thy-SO_3^- was remarkably stable in unbuffered aqueous solution: its 10^{-2}M-solution (pH *ca.* 6) can be stored at room temperature for one day without any detectable reversion into thymine. Treatment with phosphate buffer at pH 7 was found to be a very efficient means of converting Thy-SO_3^- quantitatively into thymine: heating a Thy-SO_3^- solution in 1M-sodium phosphate (pH 7) at 95 °C for 5 min completed the conversion. Since the resulting thymine can be quantified spectrophotometrically, this treatment was used routinely for the determination of concentrations of given Thy-SO_3^- solutions.

Sulphite Catalysis in the Reversion of Thy-SO_3^- *to Thymine.*—The stability of Thy-SO_3^- in water suggests that the reversion to thymine should be catalysed by sodium hydrogensulphite buffer; otherwise the equilibrium between thymine and Thy-SO_3^- in hydrogensulphite buffer must have gone completely to the product side. The reversion of Thy-SO_3^- to thymine in the presence of various concentrations of sodium hydrogensulphite is shown in Figure 1. The reversions proceeded by the pseudo-first-order rate law, and the rate was a linear function of the buffer concentration in the medium (see inset in Figure 1).

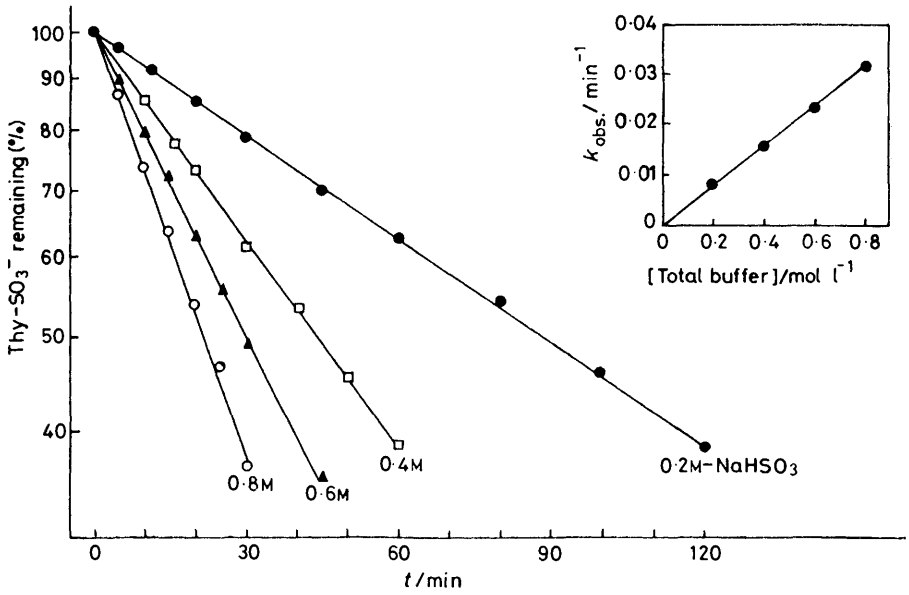


FIGURE 1 Sulphite-catalysed reversion of 5,6-dihydrothymine-6-sulphonate (Thy-SO_3^-) to thymine. Reactions were run at pH 7.0 and 25 °C. Reaction mixture contained 5×10^{-3}M-Thy-SO_3^-, sodium hydrogensulphite of indicated concentration, and an appropriate amount of sodium chloride to make the salt concentraion 1M.

The hydrogensulphite-catalysed reversal of Thy-SO_3^- to thymine was carried out at various pH values. Figure 2 shows the rate constants found (●), which indicate that the rate is very low at pH < 6, becomes higher as the pH rises from 6 to 8, and comes to a plateau at pH 8. The close fit

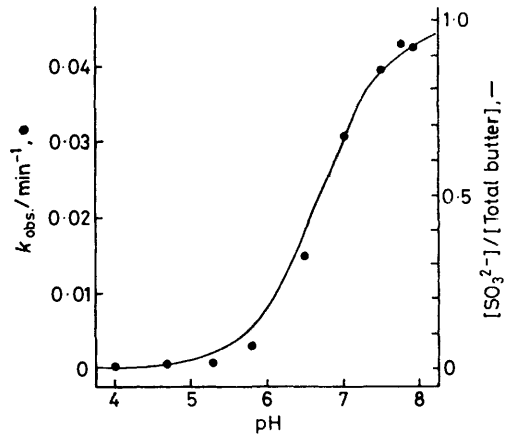


FIGURE 2 Rate of sulphite-catalysed reversion of Thy-SO_3^- to thymine as a function of pH. Reaction conditions: 0.3M-sodium hydrogensulphite, 0.5×10^{-3}M-Thy-SO_3^-, and at 37 °C. In order to fix the pH, 0.1M-sodium citrate buffer was used in the reactions at pH 4—5.8, and 0.01M-Tris-HCl in the reaction at pH 7.9. The curve drawn represents the titration at 37 °C of the hydrogensulphite buffer with NaOH, *i.e.* the fraction of SO_3^{2-} in total buffer.

between the $k_{obs.}$ values and the titration curve (with alkali) of the sodium hydrogensulphite solution demonstrates that the major reactive species is the sulphite ion (SO_3^{2-}), but not the hydrogensulphite ion (HSO_3^-).

As expected, when thymine and Thy-SO_3^- were separately treated with sodium hydrogensulphite under identical conditions, an identical equilibrium point was reached (Figure 3).

Factors Affecting the Equilibrium.—Solutions containing 10^{-2}M-thymine and 1.0M-sodium hydrogensulphite were incubated at 25 °C and the points of equilibrium were determined. In the pH range 4.5—7.5, the equilibrium shifted to the product side as the pH became lower, and the results obtained (not shown here) were essentially the same as those reported by Shapiro and his co-workers.[4] Thus, the ratio [Thy-SO_3^-] to [thymine] was a linear function of

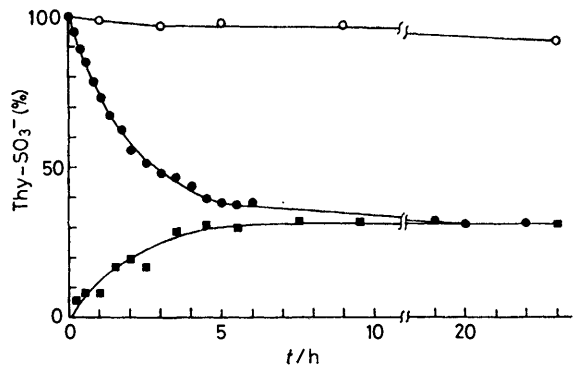


FIGURE 3 Equilibrium reached by starting either from thymine or from Thy-SO_3^-. Solutions were 10^{-2}M-thymine (or Thy-SO_3^-), 1M-sodium hydrogensulphite, and 5×10^{-2}M-sodium citrate. Incubation was at pH 6.1 and 25 °C. ■, thymine in hydrogensulphite; ●, Thy-SO_3^- in hydrogensulphite; ○, Thy-SO_3^- in 5×10^{-2}M-citrate buffer (control).

$[HSO_3^-]$. The pH-independent equilibrium constant K_E ($[\text{Thy-}SO_3^-]/[\text{thymine}][HSO_3^-]$) found in the present work was 0.61 l mol^{-1}, which was in good agreement with the value 0.62 l mol^{-1} reported by the other workers.[4] In our calculation, the pK_a value of 6.60 for the dissociation of HSO_3^- was used, which was determined by titrating

1M-sodium hydrogensulphite with sodium hydroxide at 25 °C.

The effect of temperature on the equilibrium at pH 6.2 in 1.0M sodium hydrogensulphite was investigated. The equilibrium shifted to the product side on making the reaction temperature lower. The percentage of Thy-SO_3^- in the equilibrated mixture and the time required for equilibration were 47.8%/80 h at 4 °C, 28.5%/10 h at 25 °C, and 22.4%/2 h at 37 °C.

Rate of Hydrogensulphite Addition to Thymine.—Owing to the low percentages of Thy-SO_3^- at the equilibrium, the rate of the forward reaction was difficult to measure accurately. The progress of the reactions of thymine with 0.21—1.0M-hydrogensulphite at pH 6.2 is shown in Figure 4(a). The reactions proceeded by first-order kinetics in the initial stages only; the reactions then became slower due to the reversion of accumulated Thy-SO_3^- to thymine. From the initial linear portions, the rates were estimated and were plotted against the concentrations of hydrogensulphite buffer [Figure 4(b)]. Although the apparent rate constants obtained may not be accurate, especially those at lower concentrations of hydrogensulphite, the values fit reasonably well with the line in Figure 4(b) which is drawn on the basis that the rate is proportional to the square of hydrogensulphite concentration. The second-order dependence of the forward-reaction rate on hydrogensulphite concentration is a requisite resulting from the observed first-order dependence of the reversal rate on hydrogensulphite concentration.

Comparison of the Equilibrium Constant calculated from the Rates with that found Experimentally.—The ratio [Thy-SO_3^-] : [thymine] at equilibrium should be equal to the ratio [rate constant for forward reaction (k_1)] : [rate constant for reverse reaction (k_{-1})]. Therefore, $k_1/(k_{-1}[HSO_3^-])$ should coincide with the pH-independent equilibrium constant experimentally determined. The first-order rate constant of the forward reaction, with 1.5×10^{-2}M-thymine and 1.0M-sodium hydrogensulphite at pH 6.1 and 25 °C, was determined. The rate constants found in three independent experiments were 2.91×10^{-3}, 2.55×10^{-3}, and 2.64×10^{-3} min^{-1}. The rate of the reversion of Thy-SO_3^- to thymine under identical conditions, *i.e.* at pH 6.1 and 25 °C in 1.0M-sodium hydrogensulphite, was determined. The first-order rate constants found were 5.10×10^{-3}, 5.73×10^{-3}, and 5.87×10^{-3} min^{-1} in three independent experiments. The value [average rate constant for the forward reaction]/[average rate constant for the reversal] is thus $2.70 \times 10^{-3}/5.57 \times 10^{-3}$, which is equal to 0.485. Since the pK_a of hydrogensulphite under these conditions is 6.60, $k_1/k_{-1}[HSO_3^-] = 0.485/0.76 = 0.64$ (l mol^{-1}). This value agrees well with the pH-independent equilibrium constant of 0.61 (l mol^{-1}).

Reversion of Thy-SO_3^- *to Thymine by Catalysis of Phosphate.*—The rate of reversion was measured at a pH range of 5.5—7 and at 37 °C in the presence of varying concentration of phosphate. The salt concentration was adjusted to 1.5M by addition of sodium chloride. As Figure 5 shows, the reversion was subject to catalysis by phosphate; the rate was proportional to the concentration of phosphate, and the concentration dependence was greater at higher pH values. The possible catalytic species in these reactions are HPO_4^{2-}, $H_2PO_4^-$, and OH^-; hence equation (1) obtains

$$k_{obs.} = k_{HPO_4^{2-}}[HPO_4^{2-}] + k_{H_2PO_4^-}[H_2PO_4^-] + k_{OH}[OH^-] \quad (1)$$

where $k_{HPO_4^{2-}}$, $k_{H_2PO_4^-}$, and k_{OH} represent rate constants for individual species. This equation can be expressed by equation (2) where $K_a = [HPO_4^{2-}][H^+]/[H_2PO_4^-]$. The

$$k_{obs.} = \{(\text{total phosphate})(k_{H_2PO_4^-}[H^+] + k_{HPO_4^{2-}}K_a)/(K_a + [H^+])\} + k_{OH}[OH^-] \quad (2)$$

K_a value, determined by titrating 0.3M-NaH_2PO_4 (plus 1.2M-NaCl) with 3N-NaOH at 37 °C, was 2.82×10^{-7} (or, pK_a 6.20). By employing the slopes of the two sets of reactions, *i.e.* 8.38×10^{-2} l mol^{-1} min^{-1} at pH 6.97, and 3.16×10^{-2} l mol^{-1} min^{-1} at pH 6.00, $k_{HPO_4^{2-}}$ and $k_{H_2PO_4^-}$ were calculated. The values obtained were $k_{H_2PO_4^-}$ 0, and $k_{HPO_4^{2-}}$

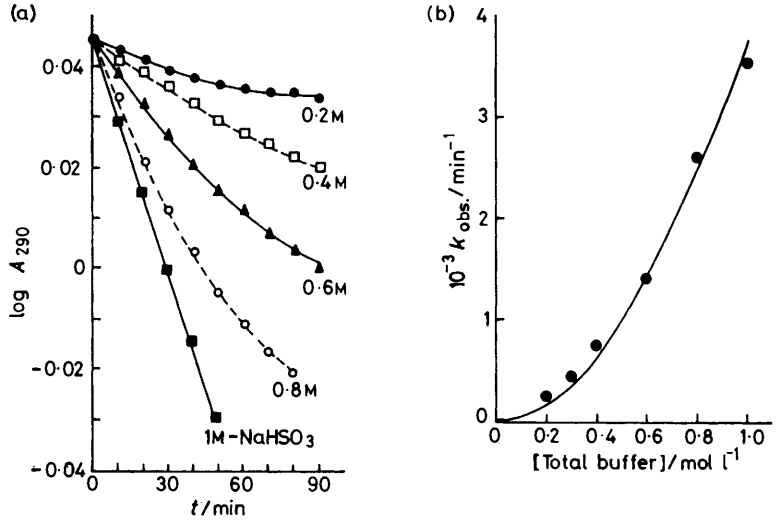


FIGURE 4 Rate of Thy-SO_3^- formation from thymine as a function of hydrogensulphite concentration. (a) Time course of the Thy-SO_3^- formation. Reaction mixtures consisted of 1.5×10^{-2}M-thymine (initial concentration), sodium hydrogensulphite of desired concentration, and sodium chloride to make the salt concentration 1M. The incubations were at 22 °C and at pH 6.2. (b) Observed first-order rate constants as a function of hydrogensulphite concentration.

0.0981 (from the pH 6.97 slope) and 0.0816 l mol^{-1} min^{-1} (from the pH 6.00 slope). From the $k_{HPO_4^{2-}}$ value (0.090; the average), the slopes at pH 6.48 and pH 5.50 were calculated and they are indicated by the broken lines in

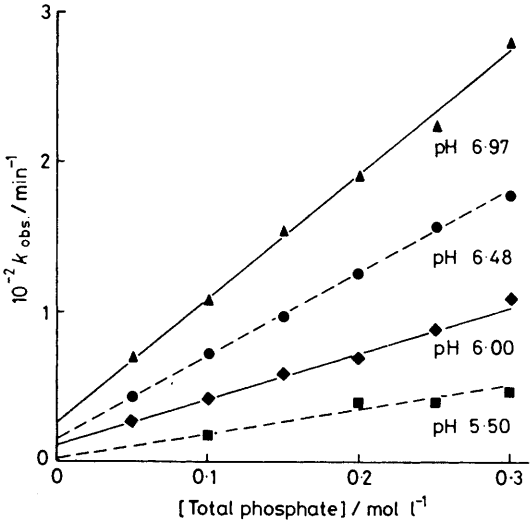


FIGURE 5 Phosphate-catalysed reversion of Thy-SO_3^- to thymine. Reaction mixtures contained 10^{-4}M-Thy-SO_3^- and sodium phosphate of desired concentration. Salt concentrations were adjusted to 1.5M by addition of sodium chloride. Incubations were at 37 °C.

Figure 5. It is seen that the lines fit well with the experimental results. Clearly, the catalytic species is HPO_4^{2-}, but not $H_2PO_4^-$.

The $k_{obs.}$ values at zero concentration of phosphate (Figure 5) can involve the term k_{OH^-}. When these $k_{obs.}$ values were plotted against the concentration of OH^- [pK_a 15.74 (ref. 8)], a linear correlation was obtained. From the slope of the correlation, the second-order rate

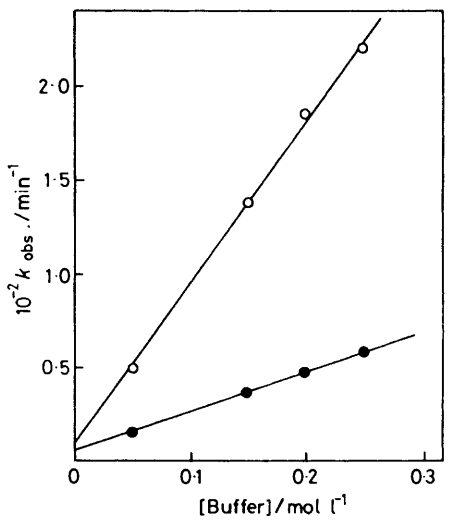


FIGURE 6 Isotope effect at position 5 of Thy-SO_3^- on the rate of phosphate-catalysed reversion to thymine. ●, [5-^{2}H]Thy-SO_3^-; ○, Thy-SO_3^-. Solutions were 10^{-4}M-Thy-SO_3^- or [5-^{2}H]Thy-SO_3^- and sodium phosphate buffer of desired concentration, pH 7.0. Salt concentrations were adjusted to 1.5M by addition of sodium chloride. Incubations were at 37 °C.

constant for the OH^- catalysis was estimated to be 2.02 × 10^4 l mol^{-1} min^{-1}.

The phosphate-catalysed reversal to thymine was carried out for Thy-SO_3^- and [5-^{2}H]Thy-SO_3^-, and the rate constants observed at several buffer concentrations are shown in Figure 6. Comparison of the two slopes indicates that the phosphate-catalysed reversion of [5-^{2}H]Thy-SO_3^- to thymine is 4.11-fold slower than that of Thy-SO_3^-. This finding suggests that the rate-determining step of the reversal is abstraction of the hydrogen at position 5 of Thy-SO_3^-.

General-base Catalysis in the Reversion of Thy-SO_3^- *to Thymine.*—The rates of reversion in several kinds of buffers were compared. For this purpose, the rates at 0.3M-buffer concentration and at 37 °C were determined. With oxygen-base buffers, the $k_{obs.}$ values were 1.93 × 10^{-2} min^{-1} in sodium phosphate at pH 6.55; 1.50 × 10^{-2} min^{-1} in sodium hydrogensulphite at pH 6.50; and 7.60 × 10^{-4} min^{-1} in sodium acetate at pH 5.97. With nitrogen-base buffers, the rates were determined at multiple pH values for each base to confirm that the bases were really the catalytic

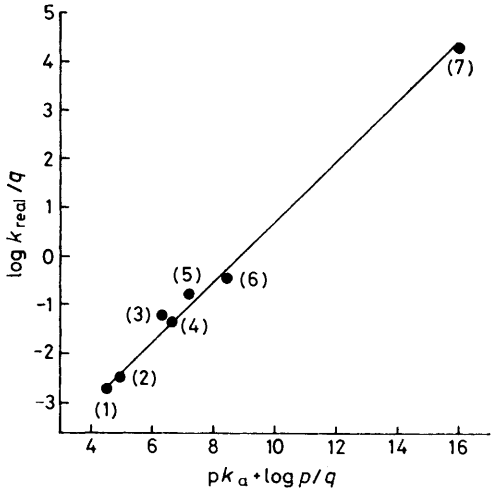


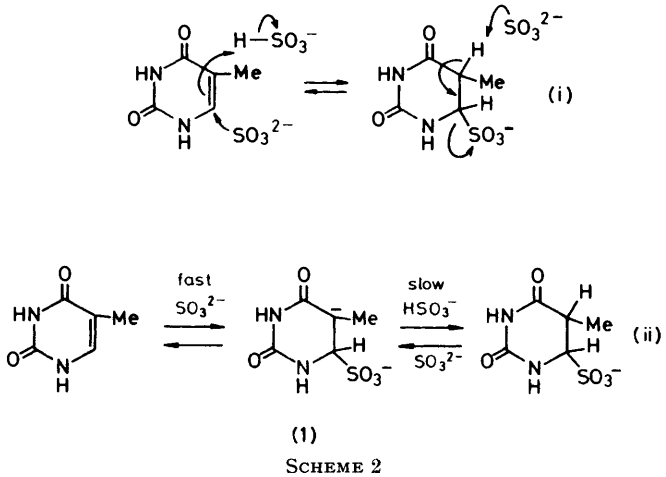
FIGURE 7 Brønsted plot of the second-order rate constants of the base-catalysed reversion of Thy-SO_3^- to thymine. The numbers in the figure correspond to acetate (1), methoxyamine (2), sulphite (3), phosphate (4), imidazole (5), tris(hydroxymethyl)aminomethane (6), and OH^- (7). The k_{real} represents the second-order rate constant, and p and q are as defined in the literature (ref. 8).

species. Thus, with 0.3M-methoxyamine at 37 °C, the $k_{obs.}$ values were 5.16 × 10^{-4} min^{-1} at pH 4.67, 5.77 × 10^{-4} min^{-1} at pH 4.94, and 9.62 × 10^{-4} min^{-1} at pH 5.46; with 0.3M-imidazole at 37 °C, they were 1.00 × 10^{-2} min^{-1} at pH 6.44, 1.63 × 10^{-2} min^{-1} at pH 6.46, and 3.08 × 10^{-2} min^{-1} at pH 6.96; with 0.3M-tris(hydroxymethyl)aminoethane at 37 °C, they were 1.23 × 10^{-2} min^{-1} at pH 6.84, 1.22 × 10^{-2} min^{-1} at pH 7.00, and 3.34 × 10^{-2} min^{-1} at pH 7.49. These $k_{obs.}$ values were then corrected for the OH^--catalysis at individual pH values, by subtracting the k_{OH^-} values that were calculated from the second-order rate constant of the OH^--catalysis. The corrected rate constants were next divided by concentrations of base in these buffers to obtain the base-catalysed second-order rate constants. The second-order rate constants (l mol^{-1} min^{-1}) thus obtained were 1.97 × 10^{-3} for acetate (pK_a 4.55), 2.63 × 10^{-3} for methoxyamine (pK_a 4.42), 1.19 × 10^{-1} for sulphite (pK_a

6.67), 8.40×10^{-2} for phosphate (pK_a 6.55), 1.12×10^{-1} for imidazole (pK_a 6.85), and 3.04×10^{-1} for tris(hydroxymethyl)aminomethane (pK_a 7.90). The pK_a values were determined by titrating the 0.3M-base solutions at 37 °C. These data, together with the rate constant for the OH^--catalysis, were subjected to a Brønsted plot.[8] As Figure 7 shows, the plot gave a linear slope with a β value of 0.60, indicating that these catalysts act as general bases to abstract the hydrogen at position 5 of Thy-SO_3^-.

DISCUSSION

Two mechanisms are consistent with the observations made for the reversible addition of hydrogensulphite to thymine. The first is the mechanism shown in Scheme 2(i). In this mechanism, SO_3^{2-} attacks as a general base



SCHEME 2

the hydrogen at position 5 of Thy-SO_3^-, and the electron displacement releases SO_3^{2-} from position 6: the rate is therefore proportional to $[SO_3^{2-}]$. In the addition to thymine, a concerted action of SO_3^{2-} and HSO_3^- produces Thy-SO_3^-; hence the rate is proportional to the product of $[SO_3^{2-}]$ and $[HSO_3^-]$, *i.e.* [total hydrogensulphite]^2.

Another possible mechanism is the two-step process of Scheme 2(ii), which is analogous to the mechanism proposed by Rork and Pitman [3] for the reversible addition of hydrogensulphite to uracil in the sense that the formation of an intermediate carbanion (1) is assumed. By this mechanism, too, the dependences of the rate to the reagent concentrations can be accounted for. Thus, the observed rate constant of the forward reaction must be proportional to the square of hydrogensulphite buffer concentration, provided that the concentration of (1) is very low, and the reverse reaction rate must be first-order in hydrogensulphite concentration.

We are unable to decide, on the information available at present, which of these mechanisms is the real one.

It is interesting to compare the reactivities of Thy-SO_3^- and 5,6-dihydrouracil-6-sulphonate (Ura-SO_3^-). Reversion of Ura-SO_3^- to uracil is also general-base-catalysed [2] and the rate-determining step is the abstraction of a proton from position 5.[2,3,9] Ura-SO_3^- is considerably more stable than Thy-SO_3^-. For example, the second-order rate constant for the tris(hydroxymethyl)-aminomethane-catalysed desulphonation of 1,3-dimethyl-5,6-dihydrouracil-6-sulphonate at 25 °C was reported to be 0.052 l mol^{-1} min^{-1} (ref. 2), whereas the rate for the desulphonation of Thy-SO_3^- by the same base at 37 °C was 0.304 l mol^{-1} min^{-1} (see above). We have measured the first-order rate constant for phosphate-catalysed desulphonation of Ura-SO_3^- at 37 °C and at pH 6.9 using 0.1—0.5M-sodium phosphate buffers. The second-order rate constant found for the HPO_4^{2-} catalysis was 1.1×10^{-2} l mol^{-1} min^{-1}, which was one order of magnitude smaller than the rate for Thy-SO_3^-.

The presence of a methyl group at position 5 of Thy-SO_3^- in place of a hydrogen atom at the same position of Ura-SO_3^- would be electronically less favourable for the release of hydrogen at position 5. The fact that Thy-SO_3^- is more labile than Ura-SO_3^- must therefore be explained in terms of other factors. There is little doubt about the *trans* nature of the hydrogensulphite addition across the 5,6-double bond of thymine, on the basis of the analogy to the hydrogensulphite addition to uracil [5,6] and on the basis of the proton coupling constant $J_{5,6}$ 6.3 Hz observed for Thy-SO_3^-, which contrasts to J 0 in the isomeric *cis*-5,6-dihydrothymine-6-sulphonate prepared by hydrogensulphite-mediated deamination of 5-methylcytosine.[7] Models of the half-chair conformation, as in 5,6-dihydrothymine,[10] show that the vicinal Me (equatorial) and SO_3^- (axial) groups are sterically close. We speculate that this proximity would produce a strain in the molecule. Thus, a reasonable explanation for the lability of Thy-SO_3^-, as compared to Ura-SO_3^-, is that the Thy-SO_3^- molecule will be relieved from the steric strain by releasing the proton from position 5 and that this situation does not exist in Ura-SO_3^-.

EXPERIMENTAL

5,6-Dihydrothymine-6-sulphonate (Thy-SO_3^-).—Thymine (315 mg) was dissolved in water (40 ml) by heating. Sodium hydrogensulphite (15.5 g) was added to the hot solution, and the pH was adjusted to 5.5 by addition of 10M-NaOH (*ca.* 1.5 ml). The solution was allowed to cool, and the mixture, in which thymine had crystallized out, was maintained at room temperature with mechanical stirring. During one day of reaction, most of the thymine went into solution. The mixture was then chilled to 4 °C and the stirring was continued for an additional 3 days. Ethanol (460 ml) was added, and the mixture was heated at 95 °C for 2 min and filtered hot. The filtrate was allowed to stand in a refrigerator overnight, and the resulting precipitate was collected by filtration. The dried solid (375 mg) was dissolved in water (1.9 ml), and the solution was heated at 95 °C, to which hot ethanol (35 ml) was added immediately. The mixture was filtered hot and the filtrate was chilled in ice to precipitate the product, which was collected and dried. This material (115 mg) was found to consist 78% of Thy-SO_3^- (corresponding to a yield of 16%) and less than 0.5% of thymine. For this estimation, a portion of this material was dissolved in water and the absorption at 264 nm (A_{264}) was measured to determine the content of thymine, and then another portion was treated with 1M-sodium phosphate buffer, pH 7.0, for 5 min at 95 °C to convert

Thy-SO_3^- into thymine, and from the resulting A_{264} value the content of Thy-SO_3^- was calculated. This material still contained *ca.* 20% sodium hydrogensulphite, but it was used for the kinetic studies without further purification, because in the kinetic studies this small amount was considered to have insignificant effects.

The procedure for preparing Thy-SO_3^- was repeated several times and the results were as follows. [Amount of thymine used as the starting material (mg)]/[amount of product obtained (mg)]/[content of Thy-SO_3^- in the product (%)]/[yield(%)]: 315/180/46/15; 315/370/48/31; 315/132/56/13; 126/104/35/16; 126/200/16/14; 126/83/50/18; 126/70/27/8. The thymine contents of these products were less than 1%. A sample for analysis was prepared by repeated precipitation from water–ethanol. It did not melt up to 250 °C (Found: C, 23.2; H, 3.8; N, 10.9; S, 12.4. $C_5H_7N_2O_5SNa\cdot1.5H_2O$ requires C, 23.4; H, 3.9; N, 10.9; S, 12.5%). $\lambda_{max.}$ end absorption; $\delta(^2H_2O)$ 1.40 (d, *J* 7.3 Hz, CH_3), 3.40 (m, 5-H), 4.54 (d, *J* 6.3 Hz, 6-H) [3-(trimethylsilyl)-propanesulphonic acid as internal standard]. On paper electrophoresis at pH 5 (0.01M-sodium acetate buffer) and at 8 V cm^{-1}, Thy-SO_3^- moved 6.2 cm toward the anode in a period of 1.5 h, while the reference compounds, uridine 5′-phosphate, uracil, and 5,6-dihydrouracil-6-sulphonate,[6] moved 4.3, 0, and 6.2 cm, respectively. On paper chromatography using ethanol–water (7 : 3 v/v) as the developing solvent, Thy-SO_3^- travelled more slowly (R_F 0.25) than thymine (R_F 0.62). The location of Thy-SO_3^- on the chromatogram was visualized by exposing the paper to ammonia vapour.

5-Deuterio-5,6-dihydrothymine-6-sulphonate ([5-^{2}H]Thy-SO_3^-).—Thymine was treated with sodium hydrogensulphite in ^2H_2O, and the product was isolated as the sodium salt by fractionating the reaction mixture on paper chromatography (solvent: ethanol–water, 7 : 3 v/v).

Kinetic Studies of the Reversion of Thy-SO_3^- *to Thymine.*—(a) *By catalysis of sodium hydrogensulphite.* 5×10^{-3}M-Thy-SO_3^- was treated with 0.2—1.0M-sodium hydrogensulphite. The incubation was at 25 or 37 °C. The salt concentrations were normalized to 1.0M by addition of sodium chloride. Portions of the solution were taken at desired intervals, diluted with 50 volumes of water to stop the reaction, and the absorbances at 264 nm against a water-reference were recorded (A_T). For complete reversion of Thy-SO_3^- to thymine, a portion was mixed with 50 volumes of 1M-sodium phosphate, pH 7, and the solution was heated at 95 °C for 5 min. The A_{264} value of this solution, against a reference of the same buffer, was then recorded (A_F). The value $(A_F - A_T)/A_F$ represented the fraction of Thy-SO_3^- in the reaction mixture. The $\log[(A_F - A_T)/A_F]$ values were plotted against the time of reaction. Linearity resulting from the first-order reaction was observed up to 50% reaction to the equilibrium. By extrapolating the linear portion, the time that should be required for conversion of 50% of the original Thy-SO_3^- into thymine (the half life) was determined. The pseudo-first-order rate constants were obtained by dividing 0.693 by the half lives. When 10^{-2}M-Thy-SO_3^- was used for the kinetic experiment, the reaction was stopped by dilution with 100 volumes of water.

(b) *By catalysis of sodium phosphate.* 10^{-4}M-Thy-SO_3^- was treated with sodium phosphate buffer of desired concentration and pH, and the reversion to thymine was determined by directly measuring A_{264} of the reaction mixture. The solutions were maintained at 37 °C by use of a thermostatted cell holder. The salt concentrations were adjusted to 1.5M by addition of sodium chloride.

(c) *Experiments for the Brønsted plot.* 10^{-2}M-Thy-SO_3^- was treated with 0.3M-buffer solutions at selected pH values and at 37 °C. The reversions were followed as described above in (a).

Determination of the Equilibrium Point.—Reaction mixtures containing 10^{-2}M-thymine, sodium hydrogensulphite of desired concentration, and 10^{-3}M-hydroquinone were incubated under nitrogen in stoppered test tubes. At appropriate intervals, portions were taken and the extent of Thy-SO_3^- formation was determined as described above. The hydrogensulphite contents were determined by iodometric titration at the end of the reactions.

Kinetics of the Hydrogensulphite Addition to Thymine.—For measuring the rate of the addition, a solution containing 1.5×10^{-2}M-thymine and sodium hydrogensulphite of the desired concentration was placed in a cuvette of 1 mm light-path, and the A_{290} was recorded against a reference containing sodium hydrogensulphite, the concentration of which was the same as that of the reaction mixture. Decrease in the A_{290} from the initial value of 1.11 represented the fraction of Thy-SO_3^- formed. The initial linear progress of the reaction (see Figure 4) was extrapolated to obtain the (hypothetical) time that should be required for conversion of 50% of the original thymine into Thy-SO_3^-. By dividing 0.693 by this half life, the $k_{obs.}$ value was obtained. The salt concentrations of the reaction mixtures were adjusted to 1.0M by addition of sodium chloride.

This work was supported by a Grant-in-Aid for Environmental Science from the Ministry of Education, Science and Culture, Japan, and a grant from the Nissan Science Foundation. We thank Dr. K. Horita of this Faculty for n.m.r. measurements.

[0/1180 *Received*, 27*th July*, 1980]

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