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Kinetics of Equilibration of Bisulfite and Dexamethasone-21-phosphate in Aqueous Solution

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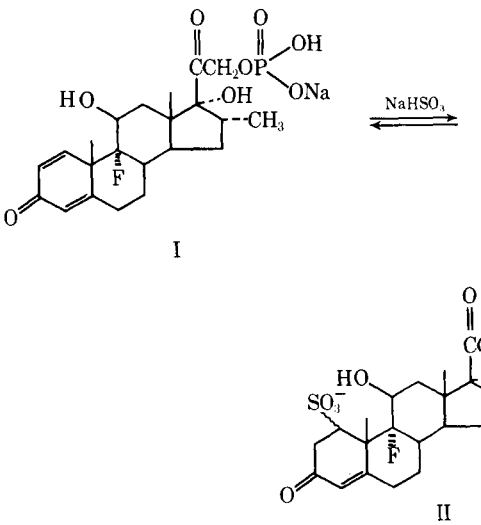
Abstract □ Dexamethasone phosphate in aqueous solution is susceptible to reversible bisulfite addition to form an A-ring-substituted sulfonic acid salt, sodium 16α-methyl-9α-fluorohydrocortisone-1-sulfonate-21-phosphate. Addition of bisulfite and dissociation of adduct proceed at comparable rates in neutral solution. The addition is a second-order reaction in which sulfite ion and monoanionic dexamethasone phosphate are the major participants; that is, a long-range effect of the steroid phosphate group on the A ring is observed. The adduct dissociation is a second-order general-base-catalyzed reaction in which dianionic adduct is more reactive than trianionic adduct. A large isotope effect was observed for dissociations of protonated and C_2-deuterated adducts. Strong dependence of equilibrium steroid composition on temperature was correlated with the temperature dependence of the ionization constant of water.

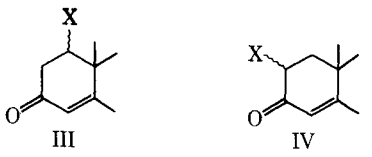
Keyphrases □ Dexamethasone phosphate and bisulfite—equilibration kinetics in aqueous solution □ Sulfite addition—aqueous solutions of dexamethasone phosphate, kinetics □ Kinetics—sulfite addition to dexamethasone phosphate in aqueous solution □ NMR spectroscopy—structure identification

Sodium dexamethasone-21-phosphate (I) enters a slow reversible reaction with sodium bisulfite, forming sodium 16α-methyl-9α-fluorohydrocortisone-1-sulfonate-21-phosphate (II) (Scheme I). This reaction is typical of conjugate sulfite addition exhibited by α,β-unsaturated ketones, *e.g.*, chalcone (1) and prednisolone phosphate (2). Since sodium bisulfite is commonly employed as an antioxidant in dexamethasone phosphate injection formulations (3), a detailed study of the kinetics and equilibria of this system was undertaken.

The structure of the adduct (II) was determined by NMR spectroscopy. The NMR parameters of the adduct are summarized in the *Experimental* section. It was evident that attack on the A ring of the dexamethasone had occurred since the signals corresponding to the vinylic protons at C_1 and C_2 were absent. The transformation involved more than simple reduction to the hydrocortisone analog, since both the C_4 and 19-methyl resonances were displaced downfield from their characteristic positions in this series. The latter observation strongly suggested that a substituent had been introduced in the A ring, most probably at C_1 in view of the large perturbation of the 19-methyl signal. This inference was supported by the presence of a $-CH_2CH-$ pattern novel to both the dexamethasone and hydrocortisone systems. It was, therefore, reasonable to attribute these signals to protons on C_1 and C_2. The chem-



Scheme I



III IV

ical shift of the methylene hydrogens (δ = 3.09 p.p.m.) indicated proximity to a functional group, such as a ketone, which favored partial structure III over IV.

The configuration of the substituent could not be assigned with assurance, since analysis of the NMR parameters led to opposing conclusions. The C_1-methine proton appeared as a triplet (ΣJ's = 11.6 Hz.), but the apparent near degeneracy of the two C_2-protons allowed no definite conclusions concerning C_1–C_2 dihedral angles[1]. The near equivalence of the C_2-methylene protons, however, suggested a β-substituent, since only then would the functional group assume the same spatial orientation with regard to both vicinal protons.

The β-configuration is also supported by analogy with the isomeric 1-acetylthio-4-androsten-3,17-dione, in which a downfield shift of the C_4-proton occurred only when the substituent was β (4). Because of spectral anomalies (*e.g.*, the C_1-methine absorbed at higher field in the equatorial configuration), however, these workers felt that the NMR data were inconclusive and they relied on rotational data for the configurational assignments. An explanation for these unexpected NMR observations was offered by Wechter *et al.* (5), who concluded from a study on a series of epimeric 1-methyl steroids that the normal chair-formed A-ring conformation was distorted in the 1β-epimers by steric interference between the 1β-methyl and 11α-hydrogen. This complicating factor, together with the fact that only one isomer was available in the present study, thus necessitates a cautious attitude on the configurational problem.

Tentative support for the assignment of the single dexamethasone adduct as the 1β-sulfonate comes from an NMR comparison study of the reactions of dexamethasone and prednisolone disodium phosphates with sodium bisulfite in aqueous medium at pH 8. Prednisolone phosphate, lacking the 9α-fluoro and 16α-methyl substituents present in dexamethasone phosphate, reacts more than 10 times faster to form two adducts, both with a new group at C_1. Dexamethasone forms only a single adduct under the same conditions, at a much slower rate. It seems most reasonable to argue that the negative end of the C–F dipole in dexamethasone significantly hinders the approach of the sulfite group on the α-side of the ring, leaving the β-sulfonate as the only product formed. With no such opposition encountered in the prednisolone case, both α- and β-sulfonates apparently form with essentially equal ease.

EXPERIMENTAL

Materials—Sodium bisulfite and other inorganic salts used were Merck reagent grade materials. Dexamethasone sodium phosphate USP XVIII was used.

Aqueous solution of dexamethasone sodium phosphate and aqueous solution of the bisulfite adduct were treated with alkaline phosphatase enzyme (6) to obtain solutions of the respective free alcohols.

Disodium 16α-Methyl-9α-fluorohydrocortisone-1-sulfonate-21-phosphate, the Bisulfite Adduct—A mixture of 10.0 g. of disodium dexamethasone-21-phosphate (0.0194 mole), 10.0 g. of sodium bisulfite, and 200 ml. of water was adjusted to pH 6.5 with 50% aqueous sodium hydroxide and heated at 60° for 24 hr. under a nitrogen atmosphere. After cooling to 5°, the reaction mixture was acidified to pH 1.5 with 96% sulfuric acid, and the resultant slurry was stirred under vacuum for 1 hr. The degassed mixture, now at pH 3.3 after loss of sulfur dioxide, was readjusted to pH 1.5 with 96% sulfuric acid and stirred under vacuum an additional 2 hr. The precipitated dexamethasone-21-phosphoric acid was removed by extraction with two 70-ml. portions of tributyl phosphate, followed by one extraction with 70 ml. of ether. The aqueous layer was readjusted to pH 1.5 and stirred under vacuum until an aliquot was free of sulfur dioxide by iodine titration (1 hr.). The remaining dexamethasone-21-phosphoric acid was removed by extraction with four 70-ml. portions of tributyl phosphate, followed by three 70-ml. portions of ether. After adjustment of the aqueous layer to pH 6.5 with 50% aqueous sodium hydroxide, the solution was freeze dried to give 17.2 g. of a solid. The solid was slurry washed three times with 40-ml. portions of methanol, and the combined filtrates were evaporated to 50 ml. This solution was clarified by pressure filtration through a filter (Seitz) fitted with an EK filter sheet. After evaporation to dryness, the residue was dissolved in 100 ml. of water and freeze dried to yield 3.5 g. of disodium 16α-methyl-9α-fluorohydrocortisone-1-sulfonate-21-phosphate.

Anal.—Calc. for $C_{22}H_{30}FNa_2O_{11}PS$: C, 44.15; H, 5.05; F, 3.17; P, 5.18. Found: C, 43.91; H, 4.57; F, 3.31; P, 5.09.

NMR (CD_3OD): δ 5.92 p.p.m. (s, 1, C_4H), 4.97, 4.62 (d, d, 2, J = 19.0, 6.5 Hz., 21-CH_2), 4.37 (s, broad, 1, $C_{11}H$), 3.85 (t, 1, ΣJ's = 11.6 Hz., C_1H), 3.09 (m, 2, 2-CH_2), 1.86 (s, 3, 19-CH_3), 1.00 (s, 3, 18-CH_3), and 0.89 (d, 3, J = 7.0 Hz., 16-CH_3); TLC as described below: single spot at R_f 0.1.

Disodium 2-Monodeutero-16α-methyl-9α-fluorohydrocortisone-1-sulfonate-21-phosphate—A mixture of 10.0 g. of disodium dexamethasone-21-phosphate (0.0194 mole), 12.6 g. of sodium sulfite (0.1 mole), and 200 ml. of deuterium oxide was adjusted to pD 6.5 with deuterosulfuric acid. The procedure already described was followed exactly, except that deuterosulfuric acid and sodium deuteroxide were employed. In comparison with the protonated adduct, the NMR was the same except for 3.85 (d, 1, J = 5.8, C_1H) and 3.09 (d, 1, C_2H); TLC: single spot at R_f 0.1.

Measurement of $pH_{1/2}$—Acid–base pH titrations of dexamethasone phosphate, the bisulfite adduct, and sodium bisulfite were performed to measure the $pH_{1/2}$'s in the neutral range. The titrations were performed at 23° under nitrogen with freshly boiled deionized water as solvent and with 0.1 *N* aqueous HCl and 0.1 *N* aqueous NaOH as titrants.

Analytical Methods—*UV Assay*—The primary analytical method used for all kinetic runs was a UV assay by which the relative molari-

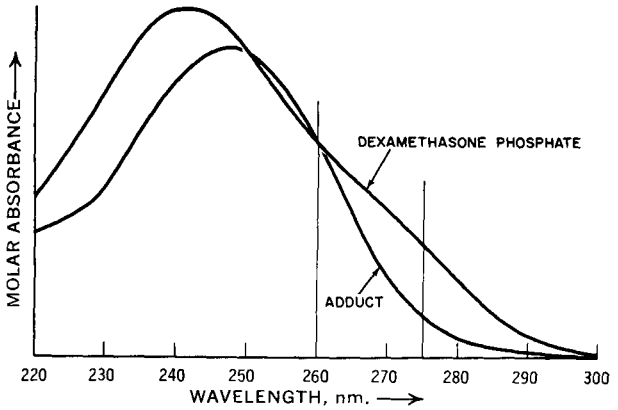


Figure 1—*UV spectra of equimolar dexamethasone phosphate and adduct in water.*

[1] This situation is not materially improved by data obtained in D_2O. In the C_2-monodeuterated analog, a coupling constant of 2.2 Hz. was observed. In the unlabeled adduct, the C_1-methine appeared as a broad, virtually structureless peak ($W_{1/2}$:10 Hz.).

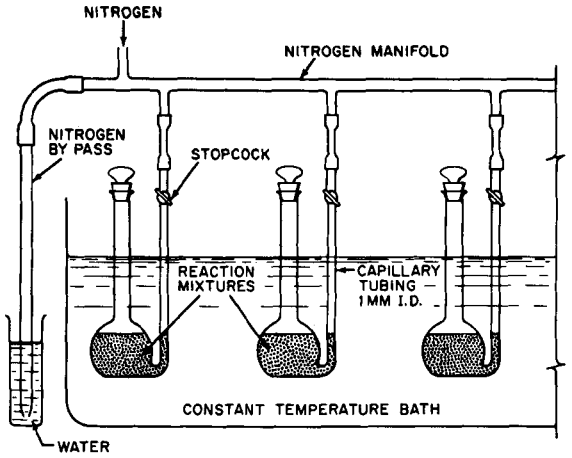


Figure 2—*Apparatus for kinetic runs.*

ties of dexamethasone phosphate and the bisulfite adduct in reaction mixtures were measured rapidly and without separation. The UV spectra of equimolar aqueous solutions of dexamethasone phosphate and bisulfite adduct are shown in Fig. 1. Both spectra are independent of pH.

There is an isosbestic point at 260 nm., established by the fact that the absorbance at 260 nm. was constant during reaction. The molar absorbance of the adduct at 275 nm., however, is much less than that of dexamethasone phosphate. The UV assay was accomplished simply by diluting aliquots of reaction mixtures with water to UV concentration and reading the absorbances at 260 and 275 nm. The relative concentrations of dexamethasone phosphate and adduct, evaluated as mole percent of total steroid present as adduct, were calculated from the ratio of the two observed absorbances.

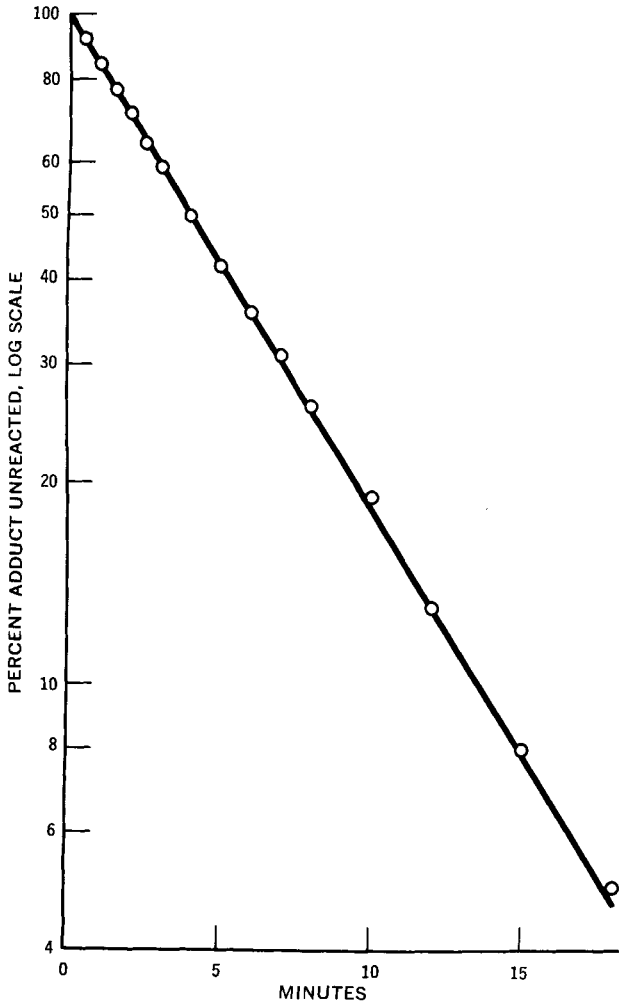


Figure 3—*Experiment 1: Dissociation of adduct in aqueous sodium hydroxide. Typical UV data, Run 1-9.*

Table I—Titration Data

Compound	$pH_{1/2}$	Ionic Strength	pKa
Dexamethasone phosphate	6.3	0.004	6.4
Bisulfite adduct	6.6	0.009	6.8
Sodium bisulfite	7.1	0.004	7.2

Bisulfite Titration—In Experiments 3 and 4, titrations were performed periodically in all runs as a check against air oxidation of bisulfite. Aliquots of reaction mixtures were added to excess standard iodine. The resulting solutions were acidified with sulfuric acid and were back-titrated with thiosulfate to the starch end-point. It was shown that dexamethasone phosphate and the bisulfite adduct did not interfere in preliminary titrations.

TLC—In Experiments 3 and 4, all reaction mixtures were examined periodically by TLC as follows. Aliquots of reaction mixtures, 2 μl., were applied directly onto silica gel GF (Analtech) plates of 250-μ thickness. The plates were developed in a saturated chamber with a solvent system of 2% v/v solution of glacial acetic acid in *n*-butanol, the latter being presaturated with water. Developed plates were viewed under short-wavelength UV light.

Procedures for Kinetic Runs—In Experiments 1 and 2, the bisulfite adduct or the deuterated adduct was the starting material. Dexamethasone phosphate and bisulfite were not present initially. Premixed aqueous sodium hydroxide or phosphate buffer, containing other added salts in some runs, was equilibrated in volumetric flasks in a constant-temperature bath at the reaction temperature (25 ± 0.05, 45 ± 0.2, 60 ± 0.2, or 75 ± 0.2°). Deionized water was used as solvent. At time zero, the preweighed adduct was dissolved; solution was complete in a few seconds.

The bisulfite formed on dissociation of adduct was not protected from air in these experiments, since bisulfite addition was negligibly slow compared to adduct dissociation. The solutions were buffered sufficiently to avoid a significant decrease in pH resulting from oxidation of bisulfite.

Timed aliquots of reaction mixtures were diluted, usually with water, to UV concentration, about 0.0002 *M*, and the UV assay was performed. Dissociation rates in neutral solutions were very slow. Accuracy in measuring reaction times for aliquots from rapid dissociations in strongly alkaline solutions was gained with the use of a pH 7 phosphate buffer as diluent.

In Experiments 3 and 4, sodium bisulfite was charged, and bisulfite addition was not insignificant in competition with adduct dissociation. It was necessary to protect reaction mixtures from air to avoid oxidation of the bisulfite. This was accomplished with the ap-

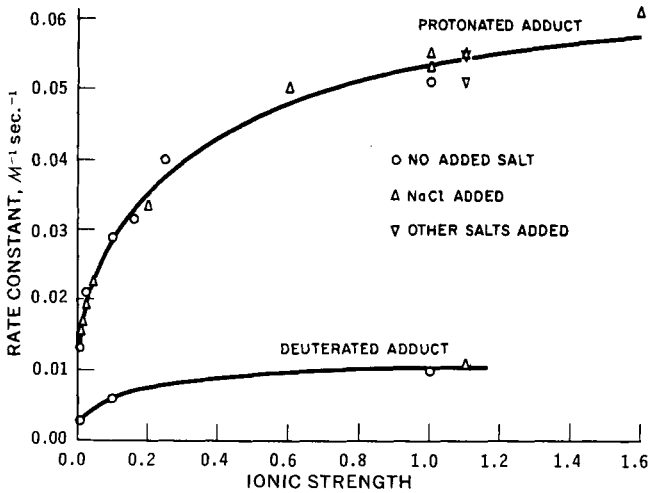


Figure 4—*Experiment 1: Dissociation of adduct in aqueous sodium hydroxide at 25°.*

Table II—Experiment 1: Rate Data for Dissociation of Adduct in Aqueous Sodium Hydroxide at 25°

Run	M_{NaOH}	Added Salt	$M_{steroid}$	Ionic Strength	Rate Constant, k, M^{-1} sec.$^{-1}$	Observed Isotope Effect, k_H/k_D
1-1	0.0048	—	0.00033	0.007	0.0130	
						4.9
1-1-D	0.0048	—	0.00033	0.007	0.00264	
1-2	0.005	—	0.00017	0.006	0.0132	
						5.0
1-2-D	0.005	—	0.00017	0.006	0.00265	
1-3	0.005	0.005 *M* NaCl	0.00017	0.011	0.0154	
1-4	0.005	0.010 *M* NaCl	0.00017	0.016	0.0167	
1-5	0.005	0.020 *M* NaCl	0.00017	0.026	0.0192	
1-6	0.005	0.040 *M* NaCl	0.00017	0.046	0.0226	
1-7	0.025	—	0.0002	0.026	0.0210	
1-8	0.1	—	0.0001	0.1	0.0288	
1-9	0.1	—	0.0002	0.1	0.0288	
						4.9
1-9-D	0.1	—	0.0002	0.1	0.00592	
1-10	0.1	—	0.0004	0.1	0.0288	
1-11	0.1	—	0.010	0.16	0.0315	
1-12	0.1	0.1 *M* NaCl	0.0002	0.2	0.0332	
1-13	0.1	0.5 *M* NaCl	0.0002	0.6	0.0501	
1-14	0.1	1.0 *M* NaCl	0.0002	1.1	0.055	
						5.1
1-14-D	0.1	1.0 *M* NaCl	0.0002	1.1	0.0108	
1-15	0.1	1.0 *M* KI	0.0002	1.1	0.051	
1-16	0.1	1.0 *M* KNO_3	0.0002	1.1	0.055	
1-17	0.1	1.0 *M* $KOCOCH_3$	0.0002	1.1	0.055	
1-18	0.1	1.0 *M* K_2CO_3	0.0002	3.1	0.077	
1-19	0.1	1.0 *M* Na_2SO_4	0.0002	3.1	0.077	
1-20	0.1	1.5 *M* NaCl	0.0002	1.6	0.061	
1-21	0.25	—	0.0002	0.25	0.040	
1-22	0.005	0.995 *M* NaCl	0.0002	1.0	0.053	
1-23	0.01	0.99 *M* NaCl	0.0002	1.0	0.055	
1-24	0.02	0.98 *M* NaCl	0.0002	1.0	0.055	
1-25	0.05	0.95 *M* NaCl	0.0002	1.0	0.051	
1-26	0.1	0.9 *M* NaCl	0.0002	1.0	0.055	
1-27	0.2	0.8 *M* NaCl	0.0002	1.0	0.055	
1-28	0.5	0.5 *M* NaCl	0.0002	1.0	0.051	
1-29	1.0	—	0.0002	1.0	0.051	
						5.1
1-29-D	1.0	—	0.0002	1.0	0.0100	

paratus shown in Fig. 2. The reaction vessels were volumetric flasks, 25 ml., each equipped with a ground-glass stopper and a capillary sidearm from the bulb to a stopcock. Several vessels containing various reaction mixtures were arranged in a constant-temperature bath and were used concurrently. The stopcocks were connected with rubber tubing to a nitrogen manifold, which included one extra port to a bypass through water. The system was purged with slow nitrogen flow throughout the experiments.

Reaction mixtures were prepared at room temperature under nitrogen by adding preweighed sodium bisulfite to solutions of known composition of dexamethasone phosphate disodium salt or bisulfite adduct in deionized water. After the addition of sodium bisulfite, the pH was adjusted by addition of concentrated aqueous sodium hydroxide. When a prepared mixture was pipeted into a reaction vessel, the stopcock of that vessel was opened. This caused nitrogen to flow through that flask, the head of water in the bypass being greater than the head in the flask. After the flask had been purged briefly, the flask was stoppered and the stopcock was closed.

Stoppers of the reaction vessels were removed, and aliquots (0.25–0.5 ml.) were taken periodically for UV assay, bisulfite titration, or TLC, or a combination electrode was inserted to measure pH. Each time a flask was opened, it was purged briefly with nitrogen by opening its stopcock.

RESULTS

pK's by Titration—The $pH_{1/2}$'s between monoanionic and dianionic dexamethasone phosphate and between dianionic and trianionic bisulfite adduct were measured by titration in water. The corresponding pK's were calculated from the equation:

$$pKa = pH_{1/2} - \frac{0.505(2Z - 1)\mu^{1/2}}{1 + 1.6\mu^{1/2}} \qquad \text{(Eq. 1)}$$

where μ represents ionic strength, and Z represents the ionic charge of the proton donor. The results are given in Table I. The steroid concentration was 0.002 *M* in both titrations. The ionic strengths calculated for half-neutralization are included in the table. Sodium bisulfite was titrated similarly, and the value 7.2 for that pKa was calculated.

Experiment 1: Dissociation of Adduct in Aqueous Sodium Hydroxide—Dissociation of the bisulfite adduct was observed in kinetic runs in which sodium hydroxide was charged in high excess to the adduct. Hydroxide concentration, therefore, was essentially constant during the reaction. By UV assay, complete dissociation of the adduct to the dienone was observed, and the dissociation was pseudo-first order; *i.e.*, plots of log (percent adduct unreacted) *versus* time were linear. Steroid compositions in aliquots of reaction mixtures were readable by the UV assay to about ±1% adduct. A typical log plot of UV data is shown in Fig. 3.

Results for Runs 1-1 through 1-29 with aqueous sodium hydroxide at 25° are summarized in Table II. The table includes composition of reaction mixtures, ionic strengths, and observed rates given as pseudo-first-order rate constants. Each rate was estimated from a plot like that shown in Fig. 3. Sodium chloride or other salts were charged in addition to sodium hydroxide and the adduct in some runs, as listed in the table. Calculated ionic strengths were nearly constant during the reaction; in cases in which the contribution of the steroid to the total ionic strength was significant, the ionic strength of the trianionic adduct was used. The letter D in a run number indicates that adduct monodeuterated at C_2 was used instead of protonated adduct. For each run with deuterated adduct, there was a similar run with protonated adduct; the observed deuterium isotope effects for those pairs of runs are included in Table II.

The two curves shown in Fig. 4 summarize the dependence of the dissociation rate at 25° on ionic strength for all the runs listed in

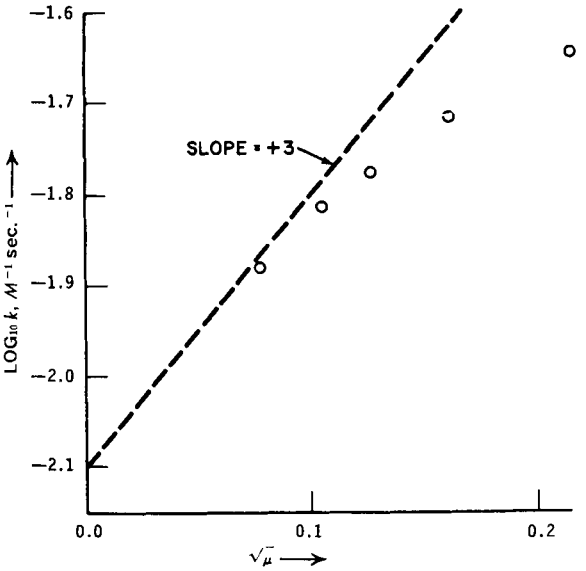


Figure 5—*Experiment 1: Dissociation of adduct in aqueous sodium hydroxide at 25°.*

Table II. In the series of Runs 1-2 through 1-6, the concentrations of sodium hydroxide and steroid were constant, and the ionic strength μ, varied by addition of sodium chloride, was relatively low. A plot of $\log_{10} k$ *versus* $\sqrt{\mu}$ for these runs is shown in Fig. 5.

In Runs 1-30, 1-31, and 1-32, initial mixtures the same as that of the 25° Run 1-1 were reacted at 45, 60, and 75°, respectively. Similar runs with deuterated adduct were performed also. The observed rates and isotope effects for these runs are summarized in Table III.

Experiment 2: Dissociation of Adduct in Phosphate Buffer—Aqueous sodium phosphate buffer solutions were prepared at several pH's and phosphate concentrations. For each run a phosphate solution was mixed with aqueous solutions of sodium chloride and adduct to make a reaction mixture with 0.0002, 0.002, or 0.1 *M* adduct and with total ionic strength of 0.1, 0.5, or 2. The ionic strengths were known accurately since the sodium chloride made the greatest contribution by far to the total ionic strength in all runs. The amount of sodium chloride used in each run was precalculated as that needed to attain the desired total ionic strength.

The results for seven series of runs at 25° are listed in Table IV. Within each series, the pH of the reaction mixtures was varied at fixed steroid molarity, phosphate buffer molarity, and total ionic strength. Series 3 was the same as Series 2, except that adduct monodeuterated at C_2 was used instead of protonated adduct.

The slow reactions of Experiment 2 were followed by UV assay for 29 days; the percentages of adduct unreacted after 29 days are listed in Table IV. The final equilibrium mixtures would contain significant amounts of adduct, especially at low pH, as can be inferred from the results of Experiment 4. In all of the runs of Experiment 2, however, the plots of log (percent adduct unreacted) *versus* time were linear through the 29-day period. The apparent dissociation rate constants, estimated from those plots, are given in the table.

Table III—Experiment 1: Rate Data for Dissociation of Adduct in Aqueous Sodium Hydroxide at Different Temperatures[a]

Run	Reaction Temperature	Rate Constant, k, M^{-1} sec.$^{-1}$	Observed Isotope Effect, k_H/k_D
1-1	25°	0.0130	4.9
1-1-D		0.00264	
1-30	45°	0.0550	4.2
1-30-D		0.0130	
1-31	60°	0.149	3.7
1-31-D		0.0404	
1-32	75°	0.379	3.2
1-32-D		0.117	

[a] Initial concentrations: 0.0048 *M* NaOH and 0.00033 *M* adduct; initial ionic strength: 0.007.

Table IV—Experiment 2: Dissociation of Adduct in Phosphate Buffer at 25°

Series	Steroid Molarity	Phosphate Molarity	Total Ionic Strength	Run	pH	Percent Adduct Unreacted after 29 Days	Rate Constant, $k \times 10^8$, sec.$^{-1}$
1	0.0002	0.02	0.1	2-1	5.85	100	—
				2-2	6.40	98	0.7
				2-3	6.85	96	1.6
				2-4	7.35	94	2.7
				2-5	7.80	88	5.1
				2-6	8.10	79	9.6
2	0.0002	0.02	0.5	2-7	5.50	99	0.2
				2-8	6.05	98	0.7
				2-9	6.55	95	2.0
				2-10	7.05	92	3.4
				2-11	7.45	86	6.0
				2-12	7.75	76	10.9
3	0.0002	0.02	0.5	2-13-D	5.50	100	—
				2-14-D	6.05	100	—
				2-15-D	6.55	99	0.5
				2-16-D	7.05	98	0.9
				2-17-D	7.50	96	1.6
				2-18-D	7.80	93	2.8
4	0.0002	0.02	2.0	2-19	5.00	98	0.7
				2-20	5.55	97	1.2
				2-21	5.95	95	2.2
				2-22	6.50	91	3.8
				2-23	7.00	87	5.7
				2-24	7.35	80	9.0
5	0.0002	0.2	2.0	2-25	5.20	98	0.9
				2-26	5.75	92	3.2
				2-27	6.20	85	6.5
				2-28	6.85	64	17.8
				2-29	7.40	46	30.9
				2-30	7.90	31	47.0
6	0.002	0.2	2.0	2-31	5.25	97	1.2
				2-32	5.75	93	3.1
				2-33	6.35	81	8.2
				2-34	6.85	64	18.1
				2-35	7.30	48	29.6
				2-36	7.65	36	41.2
7	0.01	0.2	2.0	2-37	5.25	98	1.0
				2-38	5.75	93	2.9
				2-39	6.35	81	8.6
				2-40	6.80	64	17.6
				2-41	7.20	51	27.0
				2-42	7.40	42	34.3

Experiment 3: Equilibrations at 25°—Aqueous solutions containing dexamethasone phosphate and bisulfite were equilibrated under nitrogen for 160 days at 25°. The bisulfite, used in excess in all runs, served as a buffer. Bisulfite titrations with accuracy of ±1% indicated no loss of inorganic bisulfite to air oxidation.

The only compound detected by TLC other than dexamethasone phosphate, R_f 0.3, and its bisulfite adduct, R_f 0.1, was the bisulfite adduct of dexamethasone-21-alcohol, R_f 0.4, which resulted from acid-catalyzed hydrolysis of the phosphate linkage. The content of the adduct alcohol, greater at lower pH, amounted to only about 1% of the total steroids at pH 6 after 160 days.

Initial compositions, pH data, and kinetic data for four series of runs at 25° are summarized in Table V. Apparent first-order reversible behavior was observed in all the runs by UV assay.

In Series 1, 2, and 3, the initial pH was varied with fixed initial molarities of dienone and bisulfite. Changes in ionic strength were incurred with variation of initial pH. Plots of the UV data for the first 20 days are shown in Fig. 6.

In Series 4 the initial molarities of dienone and bisulfite and the initial pH were fixed. The ionic strength was varied by addition of sodium chloride. UV data taken over a reaction time of 20 days showed that the effect of ionic strength was slight in the range of

Table V—Experiment 3: Equilibrations at 25°

Series	Initial Composition, *M* Dienone	Bisulfite	NaCl	Run	pH Initial	pH Final	Equilibrium Steroid Composition, Percent Adduct	Apparent Rate Constant, $[k_f + k_r] \times 10^7$, sec.$^{-1}$
1	0.018	0.48	—	3-1	5.95	5.95	100	5.6
			—	3-2	6.65	6.75	99	16.6
			—	3-3	7.25	7.35	90	20.8
			—	3-4	7.95	8.25	52	17.8
2	0.018	0.24	—	3-5	5.95	5.85	100	1.7
			—	3-6	6.65	6.65	96	4.8
			—	3-7	7.25	7.25	78	7.1
			—	3-8	7.95	8.20	35	8.5
3	0.036	0.48	—	3-9	5.95	6.00	100	4.7
			—	3-10	6.65	6.70	98	13.9
			—	3-11	7.25	7.45	88	18.1
			—	3-12	7.90	8.40	50	18.1
4	0.018	0.24	—	3-13	7.00	7.05	85	6.6
			0.2	3-14	7.00	7.05	—	—
			0.5	3-15	7.00	7.05	—	—
			1.0	3-16	7.00	7.05	—	—

ionic strength from about 0.5 (Run 3-13) to about 1.5 (Run 3-16). In particular, the initial addition rates for the runs of Series 4 were not measurably different.

Experiment 4: Equilibrations at 75°—Data for four series of runs at 75° are summarized in Table VI. The reaction mixtures of Series 1, 2, and 3, with dienone and bisulfite present initially, were similar to the mixtures of Series 1, 2, and 3, respectively, of Experiment 3 at 25° (compare Tables V and VI). As in Experiment 3, apparent first-order reversible behavior was observed at 75°, and exclusion of air was confirmed by bisulfite titrations.

In Series 4 of Experiment 4, the steroid present initially was bisulfite adduct rather than dienone, and both the steroid concentration and the total bisulfite content (inorganic bisulfite molarity plus adduct molarity) were the same as in Series 1. The equilibrium steroid compositions and the apparent rate constants, as functions of pH, for the runs of Series 1 and 4 were in good agreement.

After the equilibrations at 75°, the reaction mixtures of Experiment 4, Series 1, 2, and 3, were equilibrated at 60° and then at 45°. The equilibrium isotherms are shown in Fig. 7 along with 25° isotherms from Experiment 3. The observed pH of these mixtures decreased about 0.2 pH unit on cooling from 75 to 25°. Dexamethasone-21-alcohol and the adduct-21-alcohol were observed by TLC as minor components of the low pH mixtures after aging at elevated temperatures.

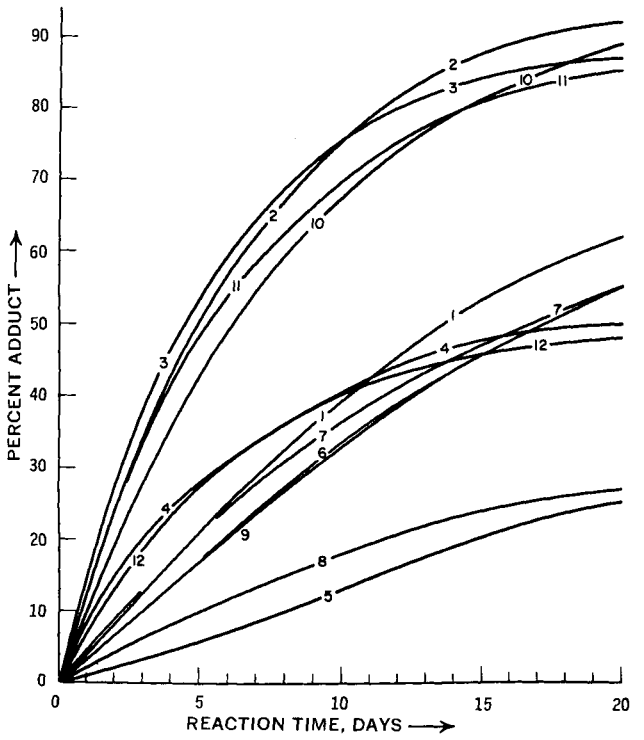


Figure 6—*Experiment 3: Equilibrations at 25°. (Graph symbols indicate run numbers as listed in Table V.)*

DISCUSSION

In aqueous solution, dexamethasone phosphate, the bisulfite adduct, and inorganic bisulfite each exist in rapid ionic equilibria as shown in Scheme II. The pKa's are 6.4, 6.8, and 7.2, respectively. Bisulfite addition to the dienone proceeds slowly, and dissociation of the adduct proceeds at a rate comparable to the addition rate. Thus, significant amounts of both dienone and adduct can be present at equilibrium.

The system is essentially free of other reactions. The only side products detected by TLC were the 21-alcohols, formed in trivial amounts by acid-catalyzed hydrolysis (see *Results*, Experiments 3 and 4).

There are two possible adduct stereoisomers, each existing in several conformations. The adduct prepared by bisulfite addition to dexamethasone phosphate, however, exhibited the kinetic behavior of a single species. That is, the essentially complete dissociations of protonated or deuterated adduct occurred with simple exponential decay (Fig. 3). This ruled out the possibility of parallel dissociations of more than one adduct species with significantly different re-

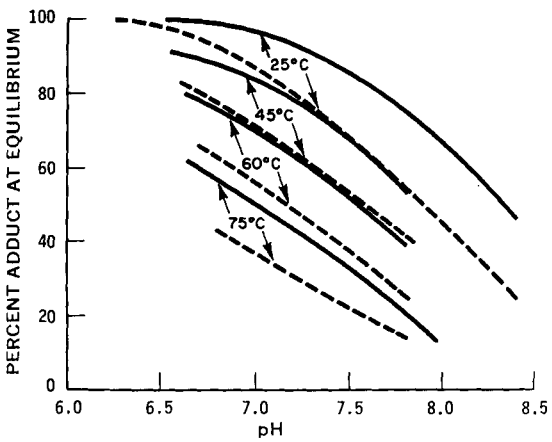


Figure 7—*Experiments 3 and 4: Equilibrium isotherms. Key: ——, 0.018 or 0.036* M *steroid, 0.48* M *sulfite; and ---, 0.018* M *steroid, 0.24* M *sulfite.*

Table VI—Experiment 4: Equilibrations at 75°

Series	Initial Composition, *M* Dienone	Adduct	Bisulfite	Run	pH Initial	pH After 2 Days	Equilibrium Steroid Composition, Percent Adduct	Apparent Rate Constant, $[k_f + k_r] \times 10^5$, sec.$^{-1}$
1	0.018	—	0.48	4-1	6.50	6.50	58	5.4
				4-2	7.00	7.35	42	9.9
				4-3	7.50	7.85	22	16.7
2	0.018	—	0.24	4-4	6.50	6.80	43	1.7
				4-5	7.00	7.30	28	3.9
				4-6	7.50	7.80	14	7.3
3	0.036	—	0.48	4-7	6.50	6.70	58	5.0
				4-8	7.00	7.20	42	8.3
				4-9	7.50	7.70	22	12.1
4	—	0.018	0.462	4-10	6.65	6.65	59	5.3
				4-11	7.50	7.40	32	9.8
				4-12	8.15	7.95	14	16.4

activities. Thus, it was inferred that the addition was stereospecific, which is also in accord with the NMR data discussed.

Dissociation of (single-species) adduct can occur by two parallel paths. C_2 of the adduct bears two protons, and cleavage of either proton leads to the same product (Scheme III). Nonstereospecific dissociation, in fact, was observed by NMR. In pH 8 phosphate buffer and in 0.1 *N* aqueous sodium hydroxide, the ratio of H to D at C_2 in the dienone produced from C_2-monodeuterated adduct was about 3:2. The dissociation rate observed by UV assay was the sum of the rates of the two paths.

The Model—Mathematical expressions of the addition and dissociation rates, consistent with the kinetic data as discussed later, are given in Eqs. 2 and 3:

$$\text{addition rate} = k_a[D^-][SO_3^{-2}] \qquad \text{(Eq. 2)}$$

$$\text{dissociation rate} = [A^{-2}]\Sigma_i k_{2di}[B_i] + [A^{-3}]\Sigma_i k_{3di}[B_i] \qquad \text{(Eq. 3)}$$

The monoanionic dienone, D^-, and the dianionic sulfite have been identified as the major participants in the addition reaction. The dissociation is general-base catalyzed, and the effects of several bases, B_i, have been observed. Dianionic adduct, A^{-2}, and trianionic adduct, A^{-3}, both participate in dissociation, and the dianion is moderately more reactive than the trianion. Each rate constant in Eq. 3, k_{2di} or k_{3di}, is the sum of the two reactivities for dissociation *via* cleavage of the two C_2 protons.

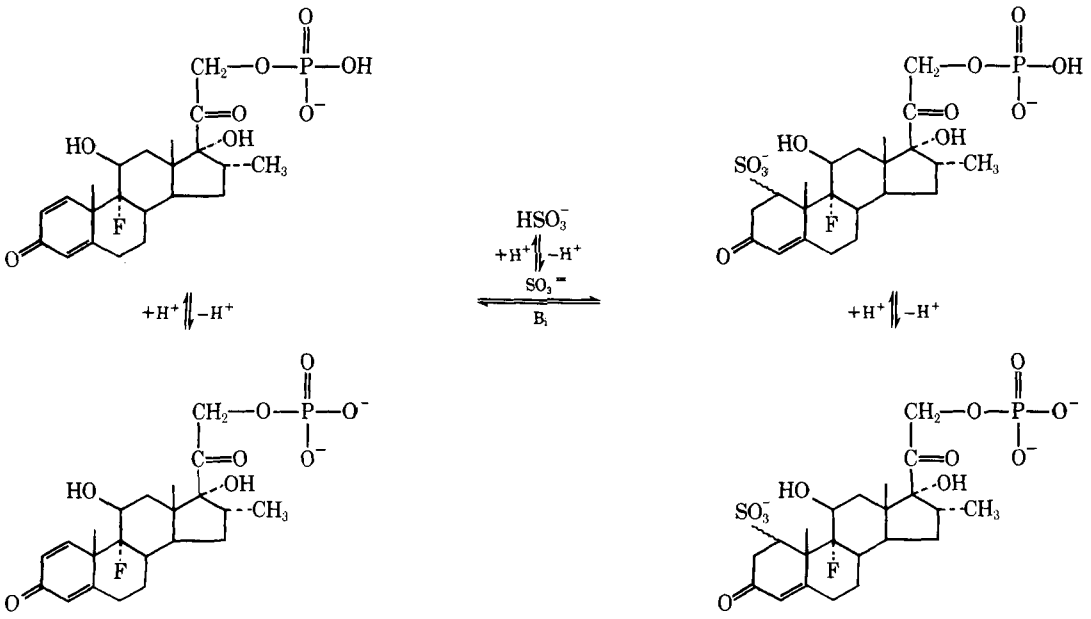
While the activities of the several reactants are calculable from the respective pK values and the observed pH, the concentrations indicated in Eqs. 2 and 3 cannot be evaluated without knowledge of the activity coefficients. Furthermore, all of the rate constants are dependent on ionic strength, and the dependence cannot be defined *a priori* except for extremely dilute solutions. For these reasons, a detailed mathematical model useful for predicting the general behavior of the system was not accessible. Under conditions of essentially constant pH and constant inorganic sulfite concentration, *i.e.*, conditions pertaining to equilibration of solutions containing sulfite in high excess to steroid, apparent first-order reversible behavior follows from Eqs. 2 and 3. Then the rate expression is given in Eq. 4:

$$\frac{dA}{dt} = k_f D - k_r A \qquad \text{(Eq. 4)}$$

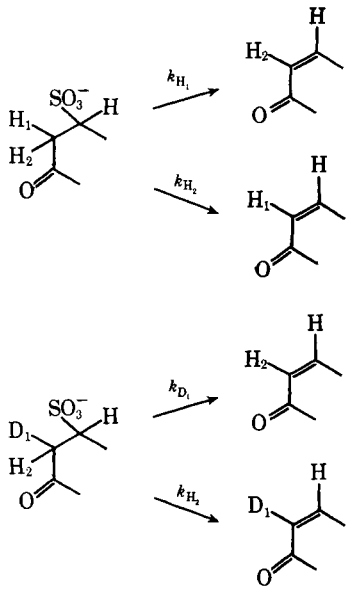
where D and A represent the concentrations of dienone and adduct, respectively, and k_f and k_r represent the apparent rate constants for addition and dissociation, respectively. Equations 5 and 6 are obtained upon integration of Eq. 4:

$$(k_f + k_r)t = \ln\left\{\frac{A_0 - (A_0 + D_0)\left(\frac{k_f}{k_f + k_r}\right)}{A - (A_0 + D_0)\left(\frac{k_f}{k_f + k_r}\right)}\right\} \qquad \text{(Eq. 5)}$$

$$(k_f + k_r)t = \ln\left\{\frac{A_0 - A_e}{A - A_e}\right\} \qquad \text{(Eq. 6)}$$



Scheme II—Reaction scheme



Scheme III—Parallel dissociation paths

where A_0 and D_0 represent initial concentrations, A represents adduct concentration at time t, and A_e represents the concentration of adduct at equilibrium.

The Dissociation—Dissociation of the bisulfite adduct in aqueous solutions containing sodium hydroxide in high excess to steroid was observed in Experiment 1. Pseudo-first-order behavior was observed, and dissociation was measurably complete at equilibrium. The rate expression, consistent with the data summarized in Table II, is:

$$\frac{d[A]}{dt} = -k_{3,OH^-}[A^{-3}][OH^-] \qquad \text{(Eq. 7)}$$

The rate constant is a function of ionic strength as well as temperature, since the reactants are ionic. (Ionic strength was constant during each run.) All of the pseudo-first-order rate constants measured in Experiment 1 for dissociations of protonated adduct at 25°, with wide variations of adduct concentration, hydroxide concentration, and ionic strength, conform to the single function of ionic strength drawn in Fig. 4. Also, the rate enhancement due to increased ionic strength was the same when various added salts were used.

Only a very small fraction of the adduct, pKa 6.8, is dianionic in aqueous sodium hydroxide, and the relative change of that small fraction is great in the range of sodium hydroxide concentration used, 0.005–1 *M*. The observed second-order rate constant was measurably independent of sodium hydroxide concentration in runs at the same ionic strength (Table II, Runs 1-22 through 1-29). This means that dianionic adduct does not contribute significantly to the rate observed; *i.e.*, the reactivity of trianionic adduct and hydroxide ion, k_{3,OH^-}, is measured in runs with aqueous sodium hydroxide.

The relation between rate constant k and ionic strength μ from the transition-state theory and the Debye–Hückel theory is:

$$\log_{10}k = \log_{10}k_0 + 1.018Z_AZ_B\sqrt{\mu} \qquad \text{(Eq. 8)}$$

where k_0 is the rate constant at infinite dilution, and Z_A and Z_B are the ionic charges of the reactants. Holding only for very dilute solutions, this equation predicts a linear dependence of $\log_{10}k$ on $\sqrt{\mu}$, the slope of the line being 1.018 times the product of the charges. The data for the aqueous sodium hydroxide runs with the lowest ionic strengths are plotted in this way in Fig. 5. The slope of the dashed line in the figure is 3. That is the slope expected at low ionic strength, since $Z_AZ_B = (-3)(-1)$, the respective charges of trianionic adduct and hydroxide ion. The kinetic data are consistent with that limiting slope.

The observed isotope effect for dissociations in aqueous sodium hydroxide at 25° was 5.0 ± 0.1 (Table II). As already mentioned, the dissociation occurs by two parallel paths, the ratio of H to D at C_2 in the dienone produced from C_2-monodeuterated adduct being

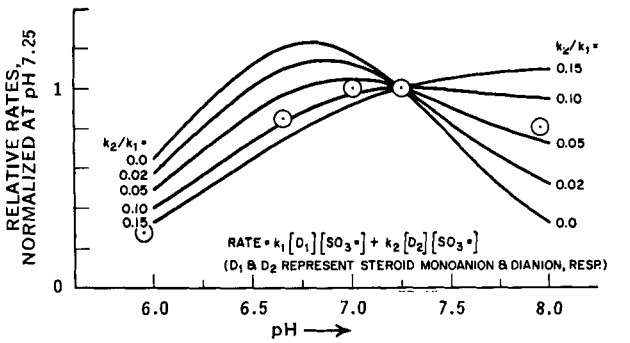


Figure 8—*Experiment 3: Simulations of the initial addition rate at 25°.*

about 3:2. The observed dissociation rate is the sum of the rates by the two paths. By numbering the C_2 protons 1 and 2 as shown in Scheme III:

$$(k_{H_1} + k_{H_2}) = 5.0(k_{D_1} + k_{H_2}) \qquad \text{(Eq. 9)}$$

and:

$$2k_{D_1} = 3k_{H_2} \qquad \text{(Eq. 10)}$$

The simultaneous equations (Eqs. 9 and 10) yield the actual isotope effect, $k_{H_1}/k_{D_1} = 7.7$, for the path involving cleavage of the isotopic bond and also yield the relative reactivity, $k_{H_1}/k_{H_2} = 11.5$, for the two C_2 protons of the protonated adduct. The large isotope effect indicates that the C_2—H bond in the activated complex is very weak.

Kinetic data for dissociations of protonated and C_2-monodeuterated adducts at several temperatures are listed in Table III. Initial concentrations and ionic strength were the same in these runs. The observed isotope effect was lower at higher temperature. The Arrhenius activation energies were 14 and 16 kcal./mole for the protonated and monodeuterated adducts, respectively. This difference of activation energies corresponds to the large isotope effect (7).

In Experiment 2, dissociation of the adduct in phosphate buffers was observed in seven series of runs. In each series the phosphate molarity and the ionic strength were fixed, and the pH was varied in the neutral range. The data are given in Table IV.

Rate enhancement due to increased ionic strength, at fixed phosphate concentration, is seen by comparison of Series 1, 2, and 4. Series 2 and 3 are similar, except that the adduct monodeuterated at C_2 was used in Series 3 instead of protonated adduct. A large deuterium isotope effect was observed, but quantitation of the effect was poor due to the small extent of dissociation.

The effect of steroid concentration was observed in Series 5, 6, and 7. The apparent rate constant, as a function of pH, was nearly independent of steroid concentration over a wide range of concentration. The slightly higher reactivity at higher steroid concentration may reflect either a difference in activity coefficients or participation of the steroids as bases.

Catalysis by phosphate was observed in Experiment 2 in Series 4 and 5, in which the ionic strengths were the same but the phosphate concentrations were different. Similarly, catalysis by phosphate at pH 11 and by carbonate at pH 9 was observed. The profile, for each series, of total apparent dissociation rate constant *versus* pH is the sum of several possible parts. Dissociation of dianionic or trianionic adduct is potentially catalyzed by any of the several bases present. With the assumption that $[OH^-] = a_{OH^-}$, the reactivity of hydroxide ion and adduct trianion measured in Experiment 1 accounts for only a few percent of the reactivity observed in neutral phosphate solutions.

The Addition—In Experiment 3, aqueous solutions containing bisulfite and dexamethasone phosphate were equilibrated at 25° (Table V). Unlike the dissociation experiments, both addition and dissociation were significant here, with bisulfite present in excess to effect convenient rates and to serve as buffer. In each of four series of runs, the initial concentrations of bisulfite and dienone were constant, and the initial pH was varied. The effect of bisulfite concentration is seen by comparison of Series 1 and 2; the effect of dienone concentration is seen by comparison of Series 1 and 3.

In the early stages of equilibration, dissociation, being first order with respect to the adduct, was insignificant. To a first approxima-

tion, the initial addition rate at a given pH was proportional to both dienone concentration and bisulfite concentration; *i.e.*, the initial rates as percent adduct per unit time were about the same in Series 1 and 3 and were lower in proportion to the bisulfite concentration in Series 2.

It was deduced from the pH dependence of the initial addition rates that the major participants in the addition are the monoanionic dienone and the dianionic sulfite. Dexamethasone phosphate and bisulfite have pKa values of 6.4 and 7.2, respectively. Therefore, the ionic compositions of both reactants depend strongly on pH in the neutral range. In each of Series 1, 2, and 3, the pH dependence of the rate was about the same, and a distinct maximum addition rate was evident at pH 7. Within the framework of Scheme II, the maximum must result from reaction of the more acidic ion of one reactant and the more basic ion of the other reactant.

The addition rates, normalized at pH 7.25, are plotted *versus* pH in Fig. 8, along with normalized calculated rate curves for reaction of SO_3^{-2}. The pKa values and pH data were used to calculate ion concentrations. The curves for several relative reactivities of monoanionic and dianionic dienone are shown, and good agreement with the data is observed for a ratio of about 0.05 for the reactivities of dianionic to monoanionic dienone. The following tests of this analysis were made, confirming that SO_3^{-2} and monoanionic dienone are the major reactants.

1. Ion concentrations in these solutions of high ionic strength could not be calculated accurately from the pK values. This was considered in simulations in which the values were varied by ±0.5 pH unit. The normalized calculated curves were shifted and the shape was altered somewhat, but a maximum comparable to that observed persisted in this test.

2. Mathematically, a maximum can result also from reaction of HSO_3^- and dianionic dienone. It is unreasonable, however, that the reactivity of HSO_3^- toward monoanionic dienone be less than that toward dianionic dienone, since the effect of the phosphate charge would be to repel the attacking HSO_3^-. There was no maximum rate in simulations with HSO_3^- as the nucleophilic agent and with the reactivity of monoanionic dienone equal to or greater than that of dianionic dienone. Instead, the calculated rate was always lower at higher pH.

3. Furthermore, lesser participation of HSO_3^- was considered, taking the reactivities of HSO_3^- toward monoanionic and dianionic dienones to be equal. The simulated maximum disappeared when the reactivity of HSO_3^- exceeded a few percent of the reactivity of SO_3^{-2} toward monoanionic dienone.

4. In each of Series 1, 2, and 3 of Experiment 3, the initial ionic strength was greater at higher pH. The reactivity, of course, was dependent on ionic strength. The described treatment of the observed initial rate–pH dependence was qualified by the results of Series 4, which showed that the ionic strength effect was insignificant. In Series 4 the initial dienone and bisulfite concentrations were the same as in Series 2, but the initial pH was 7 in all runs (Table V). The ionic strength was varied from about 0.5 to about 1.5 by addition of sodium chloride, and the initial addition rates were all measurably the same.

The greater reactivity of SO_3^{-2} toward the monoanionic dienone, deduced from the kinetic data, was evidence of a long-range effect exerted on the A ring by the charged phosphate groups. Long-range interaction was also in evidence in the pK data, the bisulfite adduct (pKa 6.8) being a significantly stronger base than dexamethasone phosphate (pKa 6.4).

Approach to Equilibrium and Equilibrium Composition—Approaches to equilibrium observed at 25 and 75°, with sulfite present in excess to steroid, were consistent with first-order reversible kinetics; *i.e.*, $\ln[(A_0 - A_e)/(A - A_e)]$ varied linearly with time (see *The Model*). The apparent rate constant was then the sum of the addition and dissociation parts ($k_f + k_r$), the former including the constant sulfite concentration as a factor and the latter being the sum of terms for various bases in general-base catalysis.

It was inferred from the initial addition rates and equilibrium compositions at 25° that hydroxide ion was the only base catalyzing dissociation in Experiment 3:

$$\text{dissociation rate} = k_{2,OH^-}(A^{-2})[OH^-] + k_{3,OH^-}[A^{-3}][OH^-] \quad \text{(Eq. 11)}$$

The participations of the sulfite and steroid ions as bases were insignificant. For each run the addition rate at equilibrium, equal to the dissociation rate at equilibrium, was calculated as the observed initial addition rate times the ratio of final to initial dienone concentrations. The equilibrium ratios of (dissociation rate)/(adduct concentration), as functions of pH, for Series 1, 2, and 3 were nearly congruent. Series 1 and 2 differed in bisulfite concentration, and Series 1 and 3 differed in steroid concentration.

Without knowledge of activity coefficients, the value of k_{2,OH^-} was estimated by Eq. 11, using pH data, pK values, and the value of k_{3,OH^-} measured in dissociations in aqueous sodium hydroxide (Experiment 1). The calculated reactivity of hydroxide with dianionic adduct, k_{2,OH^-}, was about two orders of magnitude greater than that with trianionic adduct, k_{3,OH^-}. Thus, it was deduced that a long-range effect, exerted on the A ring by the steroid phosphate group, is important in dissociation as well as in addition.

Plots of equilibrium steroid compositions *versus* pH (equilibrium isotherms) are shown in Fig. 7 for 25° (Experiment 3) and for higher temperatures (Experiment 4). The equilibrium compositions, as percent of steroid present as the adduct, were essentially independent of steroid concentration. The equilibrium was shifted toward the adduct at higher sulfite concentration and was shifted toward the dienone at higher pH, *i.e.*, at higher hydroxide concentration. The lines of percent adduct = 0 and percent adduct = 100 are asymptotes of all isotherms.

Equilibrium steroid composition is a strong function of temperature. The ionization constant of water increases markedly with temperature. At a given pH, therefore, the hydroxide-ion concentration is higher at higher temperature, and dissociation is enhanced. Small effects of temperature on the pK's of bisulfite, dienone, and adduct can be expected. In fact, the pK's for many weak electrolytes have minimum values in the range 0–60° (8). Thus, the observed shift to less adduct at higher temperature can be explained, assuming that the temperature dependences of the several rate constants are comparable. A 10-fold increase in hydroxide concentration, for example, corresponds either to an increase of 1 pH unit at a given temperature or to a temperature increase of about 35°. It is seen from Fig. 7 that both such changes of pH or temperature produce about the same equilibrium steroid composition from any chosen point on the curves shown.

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