present in the broth must be the same in both experiments. The nature of the preequilibrium process must be changed. Some investigators (40–42) studied permeation of oxytetracycline in sensitive and resistant cells at high antibiotic concentrations and found it depends upon an energy-requiring process. Franklin and Godfrey (11) verified a glucose-dependent uptake at low concentrations of tetracycline and chlortetracycline. The condition of the cells in these cultures is so different that it is hard to make meaningful comparisons. However, it seems clear that the ability to permeate the cells is an important factor in antibiotic acitivity, whether this process is an inactive or active diffusion.

SUMMARY

The kinetics of inhibition of cell division and protein and nucleic acid syntheses have been investigated for *E. coli* W cultures in the presence of a series of 18 tetracycline antibiotics. The rate constants for these parameters have been found to be linear functions of the antibiotic concentration. The proportionality constants have been found to be the same for all parameters and are reported as a measure of the activity of the analogs.

The time of onset of inhibition and the time of recovery from inhibition have been investigated; the order, protein synthesis occurring before either nucleic acid synthesis or cell division, has been observed. This order is interpreted as consistent with an inhibition of protein synthesis as the primary mode of action. The existence of a lag time for onset of inhibition of protein synthesis, its growth-rate independence, and its variation with broth pH are interpreted as due to the existence of a finite permeation time.

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Hydrolytic Degradation of Itobarbital

H. V. MAULDING and M. A. ZOGLIO*

Abstract □ The breakdown of 5-allyl,5-isobutylbarbituric acid in aqueous solution at 60, 70, and 80° in the pH range 6.7–13 has been investigated. The rate–pH profile is given and explained in terms of water or hydroxide attack on either neutral species or one or both of the dissociated forms. The rate of solvolysis was not markedly affected by phosphate or borate buffers under the conditions studied.

Keyphrases □ Itobarbital—hydrolytic degradation □ Hydrolysis—itobarbital □ pH–hydrolysis rate profile—itobarbital □ Phosphate, borate buffers, effect—itobarbital hydrolysis □ UV spectrophotometry—analysis

In the course of research concerning the stability of various pharmaceutical dosage forms, a study was undertaken of the decomposition of the barbituric acid derivative, itobarbital. The aim was delineation of the

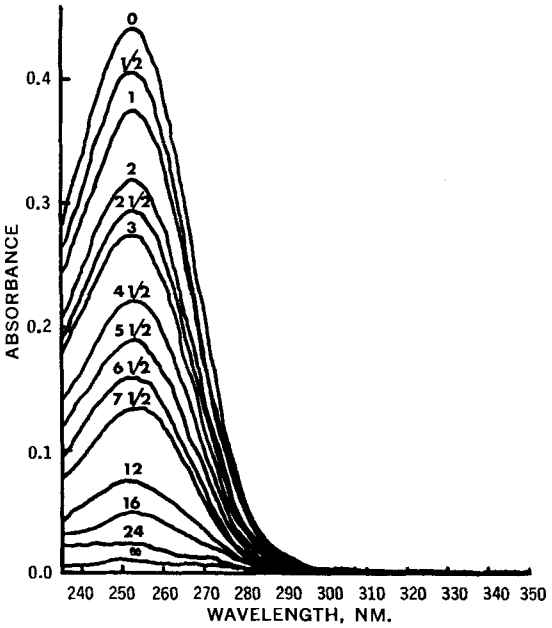

Figure 1—*Curve for absorbance diminution of itobarbital (2.2 × 10⁻⁴ M) as a function of time, hr., for 0.6 N NaOH at 60°. The number of hours after initiation of the reaction is indicated on the curves. Each sample was diluted to four times its initial volume, and these readings are shown in the graph.*

neutral and alkaline solvolysis of the compound.

Papers dealing with phenobarbital breakdown (1, 2) and other barbiturates are available (3), as well as an excellent review of barbiturate kinetics (4).

EXPERIMENTAL

Kinetic Procedures—A stock solution of sodium itobarbital[1] containing 271 mg. (0.0011 mole)/100 ml. was prepared using distilled water. Aliquots (4 ml.) were taken from the stock solution and placed in volumetric flasks (200 ml.) containing the appropriate buffer or NaOH solution (196 ml.). These flasks were previously equilibrated at the selected temperatures (60, 70, and 80 ± 0.1°), and their final concentration with regard to barbiturate was 2.2 × 10^{-4} *M*. These solutions were sampled periodically (10 ml.), and their UV spectra were read between 210 and 250 nm. on a Cary model 14 recording spectrophotometer.

Buffer solutions, unless otherwise specified, were run at an ionic strength of 0.1. Alkali solutions were prepared by dilution of standardized NaOH solution with freshly boiled distilled water and were studied at ionic strength of 0.1 wherever possible (NaCl added to bring up ionic strength).

The pH values of the buffer solutions were determined on a Metrohm pH meter standardized with borate and phthalate buffers (5) at the pertinent temperatures. The pH's of the NaOH solutions were calculated from activity coefficient data available in the literature (6). The pKa_1 values were determined at the temperature of the kinetic runs by titration of 0.005 mole 5-allyl,5-isobutylbarbituric acid with 5 ml. 0.1 *N* KOH (5).

RESULTS AND DISCUSSION

The decomposition of itobarbital over the pH range from neutrality to 13 was found to follow first-order kinetics for at least three half-lives under the experimental conditions employed. The degradation was monitored by absorbance loss of the UV chromophore as a function of time. The $\lambda_{max.}$ was located between 237 and 255 nm., depending on hydrogen-ion concentration, and a small residual absorbance remained at $t \sim \infty$. Peak height varied with pH. A linear absorbance–concentration (Lambert–Beer) relationship was exhibited at the various acidities.

The absorbance at $\lambda_{max.}$ for the pH values and temperatures studied disappears by apparent first-order processes with no perceptible change in location of the absorption band. A typical example of absorbance diminution with time may be seen in Fig. 1 and is illustrative of the general picture at the pH values and temperatures considered.

The observed first-order rate constants were calculated from the slopes of plots of the logarithm absorbance *versus* time using the expression:

$$\log(A - A_\infty) = \log(A_0 - A_\infty) - kt/2.303 \qquad \text{(Eq. 1)}$$

where A_0 is the initial absorbance; A_∞ is the residual absorbance; A is the absorbance at any time, t; and k is the observed or apparent first-order rate constant. Figure 2 is a characteristic plot of the data utilizing Eq. 1 at 60°; the rate constants for the various conditions are listed in Table I.

Rate Constant–pH Relationship—The rate–pH profile (Fig. 3) for 5-allyl,5-isobutylbarbituric acid is similar in shape to those of the closely related 5-halouridines and 5-halouracils (7, 8). Data used to construct the log *k*–pH profile are given in Table I.

Barbituric acids being diprotic, H_2A, ionize in a two-step process, producing HA^- and A^{-2} as well as two protons. This makes plausible the decomposition of the three species over the pH range 6.7–13 (pKa_1 about 7.8, 70°; pKa_2 about 11.8, 70°). However, water or hydrogen-ion attack on the neutral molecule may be generally considered negligible (7).

Degradation at pH values in the region of the pKa_2 (Figs. 3 and 4) may be accounted for by attack of water on the dianion, using the equation:

$$k''_{A^{-2}} = k''_{H_2O} f_{A^{-2}} = k''_{H_2O} \frac{Ka_2}{[H^+] + Ka_2} \qquad \text{(Eq. 2)}$$

where k''_{H_2O} is the specific catalytic constant for attack of water on the dianion; and the fraction of dianion present, $f_{A^{-2}}$, may be written in terms of hydrogen-ion concentration and the Ka_2. Equation 3 relates to Eq. 2 and expresses the equivalent hydroxyl-ion attack on the monoanion:

$$k''_{A^{-2}} = k_{OH^-}[OH^-] f_{HA^-} = k_{H_2O} f_{A^{-2}};$$
$$k'_{OH^-} = k''_{H_2O} f_{A^{-2}}/f_{HA^-}[OH^-] \qquad \text{(Eq. 3)}$$

This is a possibility because of observed ionic strength effects (Table II) in the alkaline region. However, the high ionic strengths which necessarily must be employed (Table II) give inconclusive results concerning the ion–ion interaction (9).

The observed rate constant may be expected to be reasonably constant in the pH 9.2–9.5 range (pKa_1 about 7.8), which is the area of predominant HA^- concentration with little H_2A and almost no A^{-2} (pKa_2 about 11.8). The contribution to the apparent rate constant, as seen in Fig. 3, may be described by the relationship:

$$k_{HA^-} = k'_{H_2O} f_{HA^-} = k'_{H_2O} \frac{[HA^-]}{[HA^-] + [H_2A] + [A^{-2}]} =$$
$$\frac{k'_{H_2O}}{[H^+]/Ka_1 + 1 + Ka_2/[H^+]} \qquad \text{(Eq. 4)}$$

where k'_{H_2O} is the specific constant for the attack of water on the monoanion. Equation 4 reduces to

$$k_{HA^-} = \frac{k'_{H_2O}[H^+]}{[H^+] + Ka_2} \qquad \text{(Eq. 5)}$$

when $Ka_1 \gg [H^+]$ and essentially no undissociated barbiturate is present, and it may be expressed as

$$k_{HA^-} = \frac{k'_{H_2O} Ka_1}{[H^+] + Ka_1} \qquad \text{(Eq. 6)}$$

where $[H^+] \gg Ka_2$. These two equations are demonstrative of water attack on the monoanion, and Eq. 6 is kinetically equivalent to (8)

$$k_{HA^-} = k_{OH^-}[OH^-] f_{H_2A} = \frac{k_{OH^-} k_w}{[H^+] + Ka_1} \qquad \text{(Eq. 7)}$$

Equation 7 describes the attack of hydroxyl ion on the undissociated molecule.

[1] Sandoptal Sod., Sandoz Pharmaceuticals (Ganes Chemical Works, New York, N. Y).

Table I—Observed First-Order Rate Constants[a] (k, hr.$^{-1}$) for Breakdown of Itobarbital (2×10^{-4} M) in Aqueous Solution[b]

Buffers[c]		60°			70°			80°		
$[NaH_2PO_4]$	$[Na_2HPO_4]$	pH[d,e]	Exptl.[f]	Calcd.[g]	pH[d,e]	Exptl.[f]	Calcd.[g]	pH[d,e]	Exptl.[f]	Calcd.[g]
0.025	0.025	6.80	0.00055	—	6.75	0.0017	—	6.75	0.0056	—
0.006	0.031	7.42	0.0011	—	7.40	0.0036	—	7.43	0.010	—
0.002	0.033	7.90	0.0016	—	7.90	0.0048	—	8.01	0.013	—
$[H_3BO_3]$	[NaOH]									
0.05	0.004	7.92	0.0016	—	7.88	0.0048	—	8.02	0.014	—
0.05	0.012	8.25	0.0016	—	8.15	0.0050	—	8.24	0.014	—
0.05	0.021	8.75	0.0017	0.0018	8.73	0.0055	0.0059	8.65	0.017	0.018
0.05	0.037	9.25	0.0021	0.0020	9.20	0.0069	0.0071	9.21	0.021	0.021
0.05	0.044	9.60	0.0023	0.0021	9.48	0.0078	0.0080	9.45	0.022	0.023
—	0.001[h]	9.94	0.0027	0.0037	9.73	0.0077	0.0092	9.52	0.023	0.025
—	0.003[h]	10.47	0.0058	0.0077	10.27	0.0128	0.0150	10.08	0.029	00.41
—	0.005	10.68	0.0081	0.0102	10.46	0.0179	0.0233	10.28	0.039	0.054
—	0.007	10.83	0.0092	0.0149	10.62	0.0211	0.0299	10.41	0.047	0.066
—	0.010	11.02	0.0131	0.0206	10.80	0.0271	0.0407	10.50	0.053	0.076
—	0.030	11.47	0.031	0.046	11.38	0.084[i]	0.095	10.99	0.121	0.171
—	0.050	11.61	0.046	0.060	11.51	0.111[i]	0.132	11.20	0.189	0.237
—	0.070	11.76	0.061	0.074	11.62	0.149[i]	0.157	11.33	0.244	0.288
—	0.100	11.90	0.078	0.089	11.70	0.149	0.171	11.49	0.315	0.342
—	0.200	12.18	0.114	0.116	11.97	0.230	0.231	11.75	0.498	0.468
—	0.400	12.45	0.137[i]	0.139	12.26	0.309	0.287	12.05	0.635	0.576
—	0.600	12.62	0.145[i]	0.149	12.44	0.335	0.317	12.23	0.657	0.634
—	0.800	12.74	0.157[i]	0.156	12.53	0.348	0.325	12.35	0.690	0.665
—	1.000	12.83	0.159	0.158	12.64	0.351	0.351	12.50	0.696	0.695

[a] Supplementary results in the following order: temperature; NaOH normality (k, hr.$^{-1}$) 40°; 0.75 (0.0229), 0.50 (0.0231), 0.25 (0.0223), and 0.10 (0.0145); 30°; 0.75 (0.00760), 0.50 (0.00759), 0.25 (0.00709), 0.10 (0.00524), and 0.05 (0.00384). [b] Ionic strength constant at 0.1 (NaCl). [c] $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 7H_2O$ hydrated forms used in preparation of buffer. [d] pH values for phosphate and borate buffers obtained from Metrohm pH meter calibrated with phthalate and borate buffers at indicated temperatures. [e] pH values of NaOH solutions calculated from: $pK_w - p\text{OH} = \text{pH}$ where $p\text{OH} = -\log a$ [NaOH]. The activity coefficient and pK_w values were obtained from the literature (6). [f] Rate constants reproducible within 10%. [g] Points below pH of third borate buffer not calculated. Values calculated from equations in *Results and Discussion* section and rounded off by authors. [h] First-order plots not strictly linear past two half-lives due to OH^- consumption. [i] Values for 0.04, 0.06, and 0.08 N NaOH, respectively. [j] Other runs gave values of 0.157, 0.151, and 0.123 for 0.8, 0.6, and 0.4 N NaOH, respectively, at 60°.

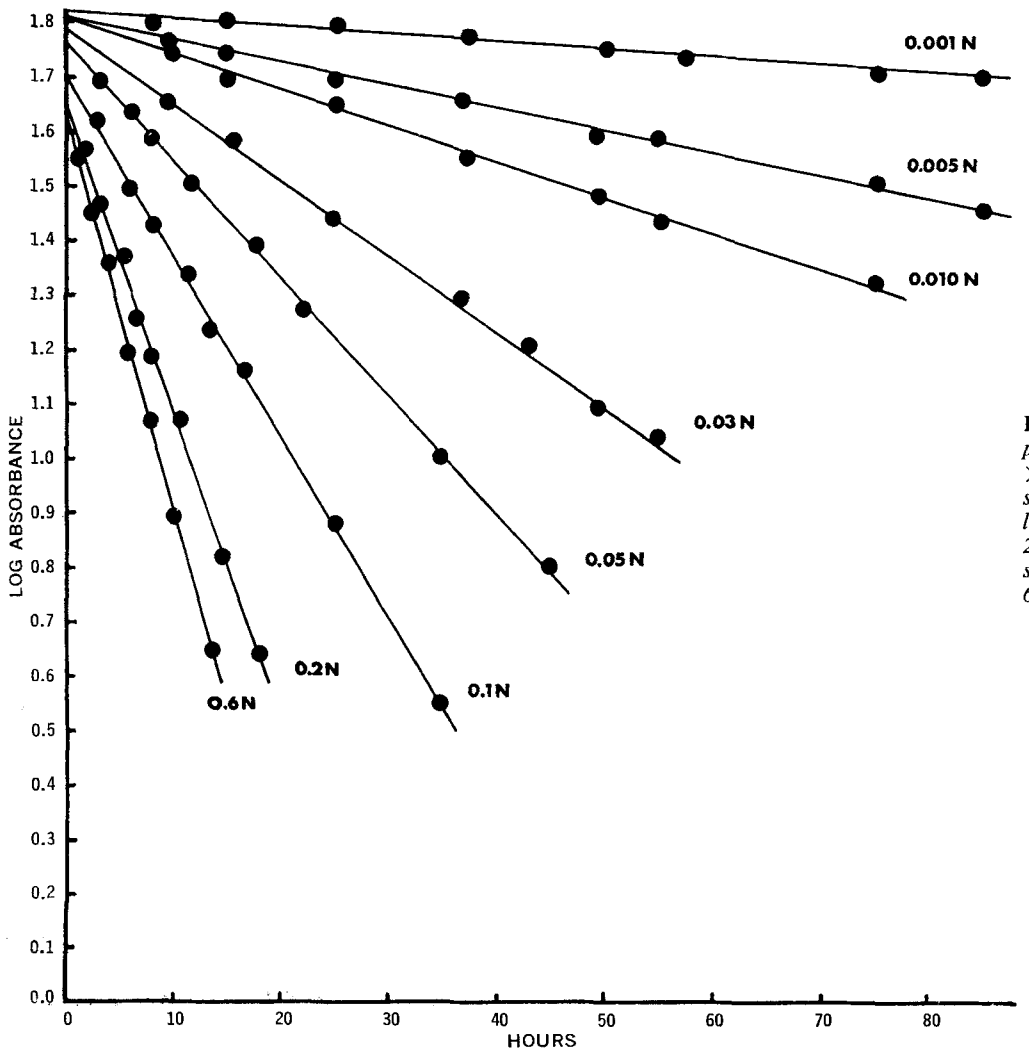

Figure 2—*Apparent first-order plots for the decomposition of 2.2 × 10^{-4} M itobarbital in NaOH solutions. Reaction followed by loss of absorbance in the range of 240–255 nm., depending on the sample normality; temperature 60°*

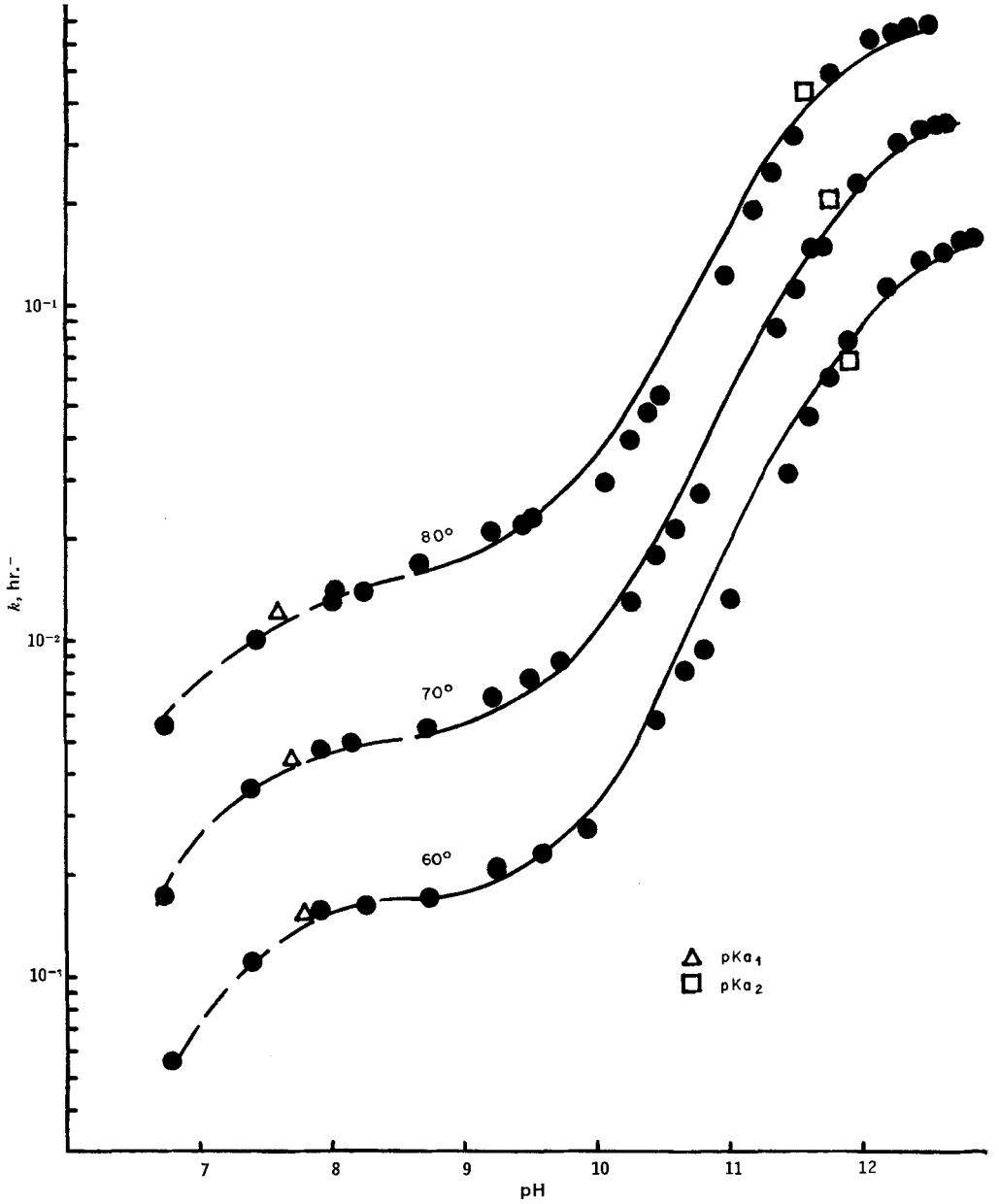

Figure 3—*The log* k–*pH profile for the solvolysis of 5-allyl,5-isobutylbarbituric acid at the indicated temperatures. Reaction monitored by loss of absorbance as a function of time.*

Figure 4 illustrates the construction of the theoretical rate–pH curve for itobarbital at 70°. The overall contribution of Eq. 2 to the log k–pH profile may be seen in Fig. 4, Curve A, and is derived in the following manner. The k''_{H_2O} is evaluated using Eq. 2 and the velocity constant data at the highest pH attainable at a given temperature—12.82 for 70° (6). It is evident in Eq. 2 that as $[H^+]$ approaches zero, $k''_{A^{-2}} \sim k''_{H_2O}$. The pKa_2 is obtained from Eq. 2 and experimental results where $[H^+] = Ka_2$; $k''_{A^{-2}} = k''_{H_2O}/2$.

The k'_{H_2O} may be similarly obtained using Eq. 4 from experimental data around pH 9.2. Since the pKa values are widely separated, Eq. 5 may be used to synthesize the right-hand limb of Curve B above pH 9 and Eq. 6 may be used for the left-hand portion with pH 6.6–9 (8). The k'_{H_2O} could also be chosen as the observed rate constant at pH 9.2 where there should be no other significant kinetic contributions.

Curve C represents the theoretical value for the rate constants and is a summation of the contributions of Curves A and B in the pH region above 8.5.

The experimental data points in Figs. 3 and 4 are listed in Table I. These data may be seen to approximate closely the theoretical rate–pH profiles described by solid lines.

The experimental results below pH 8.5 do not coincide with the left-hand portion of Curve B in Fig. 4 and are represented by dashed lines. This may be attributable to solvent or buffer attack on the undissociated species (8).

The expression describing the observed rate constant above pH 8.5 is

$$k_{obs.} = k''_{H_2O} f_{A^{-2}} + k'_{H_2O} f_{HA^-} \qquad \text{(Eq. 8)}$$

or the kinetically equivalent:

$$k_{obs.} = k'_{OH^-}[OH^-] f_{HA^-} + k_{OH^-}[OH^-] f_{H_2A} \qquad \text{(Eq. 9)}$$

A tabulation of these catalytic constants is shown in Table III.

Buffer Effects—Neither phosphate nor borate buffers exhibited appreciable catalytic activity on 5-allyl,5-isobutylbarbituric acid decomposition in the ranges and conditions employed (Table IV).

A small difference in the observed velocity constants may be noted in Table IV; however, these can be ascribed to the minor pH differences of the buffers.

Ionic Strength Effects—Table II seems to imply this phenomenon to be taking place. The question as to whether Eq. 2 or its kinetic equivalent holds for these data cannot be resolved from the results (Table II). The reason for this is that the Debye–Hückel theory and, consequently, the Brönsted–Bjerrum equation are only valid for solutions below 0.01 molal of 1-1 electrolytes (9).

Activation Energies—Estimates of the apparent activation energies were evaluated from the Arrhenius equation:

$$\log k_{obs.} = \log P - E_a/2.303RT \qquad \text{(Eq. 10)}$$

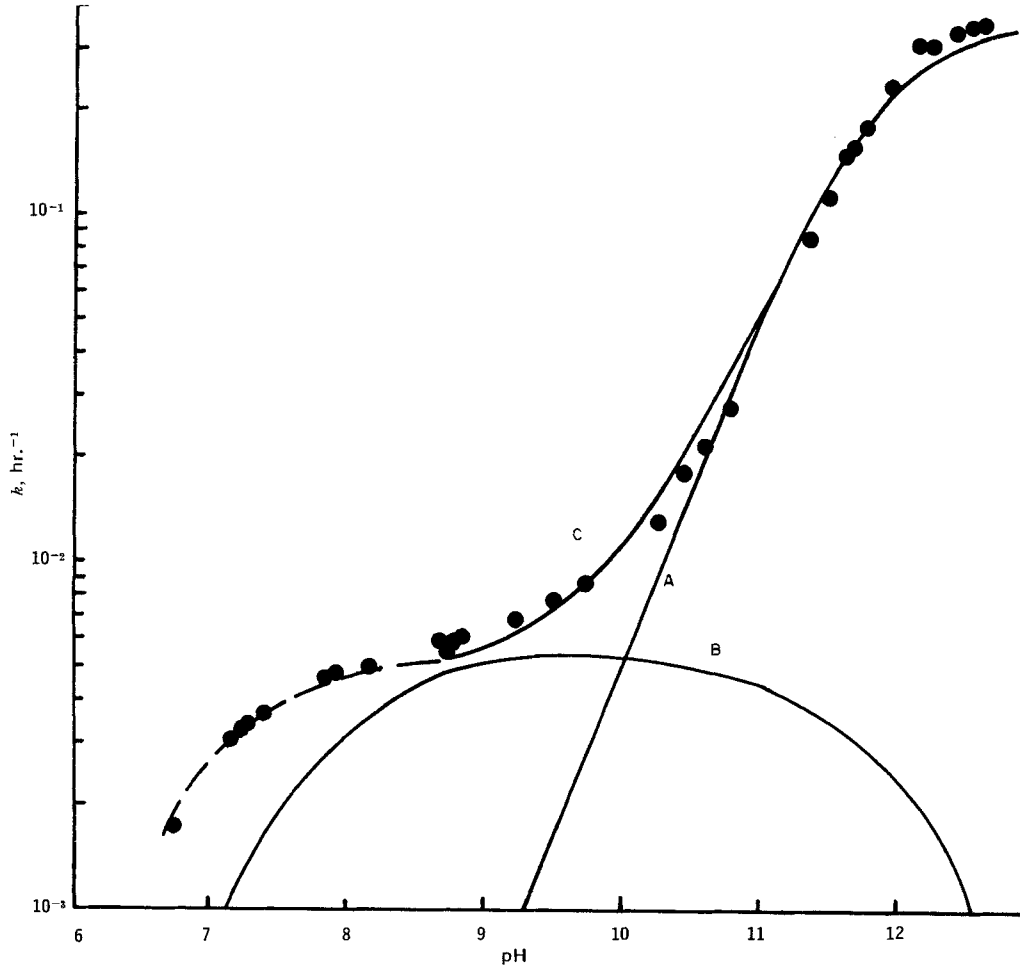

Figure 4—*Synthesis of log* k–*pH profile for itobarbital at 70°. Curve A represents water attack on dianion, and Curve B is indicative of water attack on monoanion. Curve C is a summation of A and B. See* Results and Discussion *section for a detailed explanation.*

where log $k_{obs.}$ plotted against $1/T$ absolute produces a straight line, with slope $\times$ 2.303R equal to the apparent activation energy, E_a. Values derived vary from 17.3 and 17.6 kcal./mole at 1.0 and 0.8 N NaOH to 26.6 and 27.1 kcal./mole at pH's 9.6 and 8.75, respectively.

Table II—Effect of Ionic Strength on Observed First-Order Rate Constants for Degradation of 2 $\times$ 10^{-4} M Itobarbital in 0.1 N NaOH at 70.0°

[NaOH]	[NaCl]	u	$u^{1/2}$	10^3 k, hr.$^{-1}$
0.1	0	0.1	0.32	162
0.1	0.39	0.49	0.70	187
0.1	0.90	1.00	1.00	194
0.1	1.59	1.69	1.30	194

Table III—Specific Catalytic Rate Constants (k, hr.$^{-1}$) and pKa Values for Degradation of Itobarbital in Aqueous Solution

	60°	70°	80°
pKa_1[a]	7.75	7.70	7.60
pKa_2[b]	11.90	11.80	11.60
k''_{H_2O}[c]	0.178	0.386	0.785
k'_{H_2O}[d]	0.0020	0.0063	0.020
$k_{OH^-} \times 10^2$[e]	3.70	8.41	21.49
k'_{OH^-}[f]	2.34	4.15	8.35

[a] Determined by titration of 0.005 M itobarbital at the specified temperature on a pH meter standardized by phthalate and borate buffers at specified temperature. [b] Obtained from best fit of log k–pH profiles. [c] k''_{H_2O} found using Eq. 2; pKa_2 values listed in Table III and experimental data for highest pH values of each temperature, Table I. [d] k'_{H_2O} derived from Eq. 4 using pKa values, Table III, and experimental data k_{HA^-} at various temperatures, Table I, pH 9.2. [e] Values calculated from the expression: $k'_{H_2O} f_{HA^-} = k_{OH^-} [OH^-] f_{H_2A}$ (Eqs. 4 and 7 combined). [f] Obtained from the kinetically equivalent relationship: $k''_{H_2O} f_{A^{-2}} = k_{OH^-} [OH^-] f_{HA^-}$ (Eq. 3).

SUMMARY

Itobarbital, 5-allyl,5-isobutylbarbituric acid, breaks down in aqueous solution by apparent first-order processes to produce nonchromophoric reaction products. The decomposition is easily followed in the UV by loss of absorbance as a function of time. Little buffer catalysis was witnessed under the conditions studied, and ionic strength effects in the highly alkaline range are of questionable validity.

Table I lists both experimental and calculated results, and a reasonable agreement may be noted down to pH 8.5. Below this pH, experimental and calculated values deviate, possibly due to small amounts of buffer catalysis of water attack on undissociated barbiturate. The observed rate constant may be described in the

Table IV—Influence of Buffers on Observed First-Order Rate Constants for Breakdown of 2 $\times$ 10^{-4} M Itobarbital at 70.0°[a]

pH[b,c]	Buffers: [$NaH_2PO_4 \cdot H_2O$]	Buffers: [$Na_2HPO_4 \cdot 7H_2O$]	NaCl	10^3k, hr.$^{-1}$
7.18	0.030	0.155	0	3.4
7.15	0.018	0.093	0.20	3.4
7.10	0.012	0.062	0.30	3.2
7.07	0.006	0.031	0.40	3.0
	[NaH_4BO_4]	[H_5BO_4]		
8.85	0.30	0.03	0	6.3
8.80	0.20	0.20	0.1	5.9
8.75	0.10	0.10	0.2	5.9
8.75	0.05	0.05	0.25	5.9

[a] Ionic strength constant at 0.5 for phosphate buffers and 0.3 for borate buffers. [b] pH values measured at 70° by pH meter standardized at this temperature with phthalate and borate buffers and are $\pm$ 0.05. [c] pH's of solutions at 25° were 9.30 and 7.45 ($\pm$0.05), respectively.

following manner to fit the experimental findings:

$$k_{obs.} = k''_{H_2O} f_{A^{-2}} + k'_{H_2O} f_{HA^-} \quad \text{(Eq. 8)}$$

Or it may be written in the kinetically equivalent:

$$k_{obs.} = k'_{OH^-}[OH^-] f_{HA^-} + k_{OH^-}[OH^-] f_{H_2A} \quad \text{(Eq. 9)}$$

The specific catalytic rate constants for Eqs. 8 and 9 are given in Table III along with other pertinent data.

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Competitive Binding of Two Sulfas and Penicillin G to Bovine Serum Albumin Using NMR Techniques

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Abstract □ NMR spectroscopy was employed to examine the competitive binding of sulfamerazine, sulfacetamide, and penicillin G to bovine serum albumin. Variations in spin-spin relaxation rates were measured, and evidence for the displacement of penicillin by sulfa drugs was obtained.

Keyphrases □ Sulfonamide binding—bovine serum albumin □ Penicillin binding—bovine serum albumin □ Bovine serum albumin—sulfonamide displacement, bound penicillin □ NMR spectroscopy—analysis

The phenomena of drug–protein binding and of the competitive binding of drugs for available protein sites have been the subjects of many investigations (1). In this respect the antibiotics and sulfonamides have received considerable attention. Certain of these medicinal drugs have been shown to bind to bovine serum albumin (BSA). However, the methods of investigation have varied, and the data from competitive binding in certain instances have been inconclusive. Recent studies established the value of nuclear magnetic resonance (NMR) spectroscopy as a powerful tool for conformational determinations of pharmacologically active molecules in solution (2) and for elucidation of possible interactions between small molecules (3, 4). Furthermore, NMR is invaluable in assessing the extent to which various functional groups on small drug molecules participate in drug–protein interactions (5).

Fischer and Jardetzky (6) and Jardetzky and Wade-Jardetzky (7) utilized the extreme sensitivity of spin-spin ($1/T_2$) and spin-lattice ($1/T_1$) relaxation rates to small variations in the molecular environment of a proton species to show clearly how measurement of these parameters reveals formation of intermolecular complexes. In studies on penicillin and sulfa drug binding to BSA, they demonstrated that there were changes in relaxation rates large enough to be measured. This was true in spite of the fact that the concentration of bound molecules did not exceed 1% of the total drug concentration. The ability to detect the differences in relaxation rates upon binding, especially those differences due to diminished molecular motion, particularly rotational motion, made these studies possible. Observation of the relatively large increments in relaxation rate of the phenyl protons of penicillin and the *p*-aminobenzenesulfonamide moiety of sulfonamides lead them to conclude that these portions of the drug molecules are involved in binding.

In another report, Jardetzky and Wade-Jardetzky (8) indicated that there was no displacement of sulfa drugs bound to BSA by penicillin at reasonable concentrations of penicillin. This led them to postulate that the sites of binding on BSA for sulfa drugs and penicillin were different. However, there was no indication of concentration ratios used nor was there any indication that ionic strength and/or pH were controlled. Later, Kunin (9), using dialysis studies, showed that sulfa drugs in general displace certain BSA-bound penicillins.

Jardetzky and coworkers (8) reported that no displacement of sulfa drugs bound to BSA by penicillin occurs; Kunin (9), using different techniques, observed that the reverse displacement does take place. Thus, it appeared desirable to examine both displacement avenues while, at the same time, controlling factors such as ionic strength and pH which are known to be of significance in protein-binding studies. In this paper, the authors report the results of investigations of competitive binding to BSA between sulfamerazine (I) and penicillin G (II) and between sulfacetamide (III) and penicillin G (II) while ionic strength and pH are controlled.