2-Methyl-3-homopiperidinopropionic Acid (*VII*b)—This acid, m.p. 100°, was prepared in the same way as the acids just mentioned. IR (KBr): 2400–2120 (broad, ν $\overset{+}{N}H$); 1620, 1450 (ν CO_2^-) cm.$^{-1}$. PMR (D_2O): 69 Hz. (d, 3, $J=6.5$, CH_3CHCO); 105 Hz. (s, 8, four ring CH_2 groups remote from N atom); 138–181 Hz. (m, 7, $\begin{matrix}CH_2\\CH_2\end{matrix}\!\!>\!NCH_2CH$). Mass spectrum: (percent relative abundance) *m/e* 185 (6.9%), 112 (100%).

Anal.—Calcd. for $C_{10}H_{19}NO_2 \cdot 0.5\ H_2O$: C, 61.8; H, 10.3. Found: C, 61.7; H, 10.6.

3-Ethylaminopropionic Ethylamide (*V*)—In an attempt to prepare methyl 3-ethylaminopropionate by reacting ethylamine and methyl methacrylate at room temperature for 10 days, using the general method for the preparation of esters, the reaction mixture was fractionally distilled and yielded the title compound (46% yield), b.p. 113–114°/2 mm. IR (film): 3300 (ν NH); 1645 (ν C=O) cm.$^{-1}$. PMR (CCl_4): 63 Hz. (t, 3, $J=7$, $CH_3CH_2NHCH_2$) overlapping with 66 Hz. (t, 3, $J=7$, $CONHCH_2CH_3$); 88 Hz. (s, 1, CH_3CH_2NH); 498 Hz. (s, 1, CONH), both singlets exchanged in D_2O.

Anal.—Calcd. for $C_6H_{14}N_2O$: C, 55.3; H, 10.8. Found: C, 54.9; H, 10.8.

*2-Methyl-3-*n*-propylaminopropionic* n-*Propylamide* (*VI*)—The title compound was obtained in 40% yield in a manner similar to that described for the preparation of V.

This compound was characterized as the hydrochloride, m.p. 130–131.5°. IR (mineral oil) 3270 (amide ν NH); 2900–2410 (ν $\overset{+}{N}H_2$); 1660 (C=O).

Anal.—Calcd. for $C_{10}H_{23}ClN_2O$: C, 53.92; H, 10.41. Found: C, 53.83; H, 10.41.

REFERENCES

(1) R. T. Coutts, K. K. Midha, and K. Prasad, *J. Med. Chem.*, **12**, 940(1969).

(2) R. A. Hughes, U. S. pat. 2,965,671 (1960); through *Chem. Abstr.*, **55**, 16423e(1961).

(3) S. I. Suminov, *Vestn. Mosk. Univ., Ser II*, **22**, 75(1967); through *Chem. Abstr.*, **67**, 21425r(1967).

(4) P. Bieber, *Ann. Chem.*, **9**, 674(1954).

(5) L. W. Reeves, *Trans. Faraday Soc.*, **54**, 314(1958); **55**, 1684 (1959).

(6) J. H. Beynon, "Mass Spectrometry and its Application to Organic Chemistry," Elsevier, Amsterdam, The Netherlands, 1960, pp. 391–393.

(7) A. Vystrcil and S. Hudecek, *Chem. Listy*, **44**, 262(1950).

(8) D. R. Howton, *J. Org. Chem.*, **10**, 277(1945).

(9) S. David and P. Sinay, *Bull. Soc. Chim. Fr.*, **1965**, 2301.

(10) British pat. 1,018,810 (1966); through *Chem. Abstr.*, **64**, 12860a(1966).

(11) British pat. 1,008,177 (1965); through *Chem. Abstr.*, **64**, 5238f(1966).

(12) A. H. Beckett, A. F. Casy, and G. Kirk, *J. Med. Pharm. Chem.*, **1**, 37(1959).

(13) V. J. Traynelis and J. G. Dadura, *J. Org. Chem.*, **26**, 686 (1961).

(14) P. A. Cruikshank and J. C. Sheehan, *J. Amer. Chem. Soc.*, **83**, 2891(1961).

(15) B. V. Matveev and G. G. Tsybaeva, *J. Gen. Chem. USSR*, **34**, 2512(1964).

(16) K. K. Midha, R. T. Coutts, J. W. Hubbard, and K. Prasad, *Eur. J. Pharmacol.*, **11**, 48(1970).

(17) U. S. pat. 3,251,875 (1966); through *Chem. Abstr.*, **65**, 3758d(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 13, 1970, from the *Faculty of Pharmacy and Pharmaceutical Sciences*, and the *Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada.*

Accepted for publication July 20, 1970.

Abstracted in part from a thesis by K. K. M. in partial fulfillment of the Doctor of Philosophy degree requirements.

This research was supported by the Defence Research Board of Canada (Grant No. 8875-05) and by the Medical Research Council of Canada (Grant No. MA-2993).

The authors acknowledge the assistance of Mrs. A. G. MacArthur in this project.

Structure–Activity Relationships of Tetracyclines I: Inhibition of Cell Division and Protein and Nucleic Acid Syntheses in *Escherichia coli* W

G. H. MILLER*, S. A. KHALIL†, and A. N MARTIN‡

Abstract □ The kinetics of inhibition of *Escherichia coli* in a peptone broth as a function of the concentration of 18 tetracyclines were determined. Viable and total cell count methods were used to measure rates of cell division. Rates of protein and nucleic acid syntheses were determined simultaneously by a membrane filter technique in conjunction with the Folin–Lowry assay and the orcinol reaction, respectively. The relationship of these rate constants to antibiotic concentration is an accurate estimate of activity under the test conditions. Different times before onset of inhibition of protein synthesis, cell division, and nucleic acid synthesis are observed in kinetic experiments. These differences are interpreted as being consistent with theories of primary inhibition of protein-synthesizing systems.

Keyphrases □ Tetracyclines—structure–activity relationships □ Structure–activity relationships, tetracyclines—*E. coli* inhibition □ Rate constants, *E. coli* cell division—tetracyclines □ Protein, nucleic acid syntheses, *E. coli*—tetracycline effect □ Viable, total cell counts—*E. coli* division rates □ Concentration, tetracyclines—*E. coli* inhibition

Quantitative biological activities of a large number of compounds obtained under identical conditions in a precise manner are required to establish structure–activity relationships. Ideally, these activities should be directly related to the mechanism of action of the compounds and be free of extraneous competing equilibria. Presently available activities for tetracycline antibiotics were summarized by Barrett (1). Many of these activities were determined under conditions such that the results parallel clinical activity, a type of activity

Table I—First-Order Generation-Rate Constants, sec.$^{-1}$ × 10^4, for *E. coli* W Cultures Inhibited by Various Tetracycline Antibiotics, $M \times 10^6$

M	k_v	k_p	$k_{n.a.}$
	Tetracycline		
0.000	5.57	5.85	5.07
0.599	3.80	4.46	3.85
0.898	3.23	2.97	2.89
1.198	2.27	1.96	2.10
1.497	1.49	1.76	1.45
1.796	1.10	1.25	1.27
	7-Chlortetracycline		
0.000	5.41	5.94	5.80
0.246	4.82	4.51	4.97
0.492	3.48	3.33	3.53
0.788	2.33	2.17	2.14
0.985	2.16	1.84	2.16
1.231	0.52	0.93	0.51
	5-Hydroxytetracycline		
0.000	5.75[a]	5.79	5.97
0.537	4.71	3.49	3.75
0.878	4.04	3.47	—
0.878	3.75[b]	—	—
1.073	2.25	2.24	2.14
1.376	2.23	2.24	2.40
1.376	2.20[b]	—	—
	7-Chloro-dm-tetracycline[c]		
0.000	5.42	5.02	5.90
0.221	4.99	4.41	5.26
0.441	3.37	3.05	3.68
0.706	1.46	1.13	1.50
0.882	0.55	0.68	1.06
1.103	—	0.55	0.59
	dm-do-Tetracycline[d]		
0.000	5.89	5.05	5.25
0.978	4.58	4.55	4.77
1.955	3.65	3.42	3.50
2.972	2.76	2.57	2.47
3.910	1.46	1.57	1.65
4.888	0.84	1.07	0.97

M	k_t[e]	k_p	$k_{n.a.}$
	7-Bromo-dm-do-tetracycline		
0.000	5.89	5.41	5.89
2.468	4.29	3.78	3.52
4.938	2.70	2.59	2.29
7.406	1.26	1.50	1.50
9.876	—	0.85	0.55
	7-Chloro-isotetracycline		
0.00	5.71	5.26	5.40
163.49	5.81	4.74	5.39
322.30	5.71	4.86	5.53
	12*a*-Deoxytetracycline		
0.00	5.71	5.26	5.40
79.14	3.90	3.69	3.59
156.03	1.59	1.57	0.95
	5*a*(11*a*)-Dehydrochlortetracycline		
0.00	5.71	5.26	5.40
150.68	5.23	4.26	4.55
297.06	4.64	4.03	4.66
	2-Cyano-2-decarboxamidotetracycline		
0.00	5.71	5.26	5.40
33.16	5.94	4.92	5.44
128.88	5.88	4.63	4.72

[a] An average value, determined on several different days. [b] Values of k_t which were used in the calculation of the viable inhibitory rate constant (Table III). [c] dm- is 6-demethyl-. [d] dm-do- is 6-demethyl-6-deoxy-. [e] Values of k_t were obtained with 0.2% HCHO in the diluting solution (see *Experimental*).

that may be different than activity at the receptor site. Different activities were reported for several analogs under various test conditions. The present work was undertaken to establish activities suitable for the study of structure–activity relationships, insofar as possible, for a series including both clinically active and "inactive" tetracycline antibiotics.

Activities were obtained by three types of kinetic measurements on exponentially growing *Escherichia coli* inhibited by the antibiotics. Rate constants for cell division, $k_{c.d.}$, in a high peptone broth were measured by the viable plate count method of Brown and Garrett (2) and by the total cell count method of Garrett and Miller (3). A filter technique using detergent-extracted cellulose ester membranes was developed to isolate trichloroacetic acid (TCA)–ethanol precipitates of the *E. coli* as a function of time of growth. The hot TCA-soluble extract of these precipitates was used to measure nucleic acids by the orcinol reaction. The hot TCA-insoluble material remaining on the filter was trypsin-digested, and the protein content was estimated by the Folin–Lowry assay. These data were used to calculate rate constants for protein synthesis, k_p, and nucleic acid synthesis, $k_{n.a.}$.

Inhibition of protein synthesis and nucleic acid synthesis, rather than inhibition of cell division alone, was studied so that the activities might be more directly related to the mode of action. Gale and Folkes (4) showed that protein synthesis in washed suspensions of *Staphylococcus aureus* is inhibited at concentrations of tetracyclines similar to those causing inhibition of growth.

Nucleic acid synthesis was reported to be inhibited only at higher antibiotic concentrations. Hash *et al.* (5) reported that incorporation of several radioactive amino acids into cytoplasmic protein was greatly inhibited by chlortetracycline and tetracycline at 1 mcg./ml. Chemical analysis of inhibited cells revealed that the cells contained increased cell wall content and hexosamine, as well as ribonucleic acid (RNA), but had decreased protein and deoxyribonucleic acid (DNA) content as compared with control cells. Cerny and Habermann (6) made kinetic measurements of the inhibition of protein and nucleic acid syntheses in cultures of *E. coli* C at tetracycline concentrations of 20–50 mcg./ml. They found an immediate cessation of protein synthesis followed by a later but complete inhibition of nucleic acid synthesis. Holmes and Wild (7) recently discussed the nature of an RNA synthesized in unaerated *E. coli* K12 cultures whose protein synthesis was inhibited by 15 mcg./ml. of chlortetracycline. These authors felt that ribosomal precursor RNA accumulates under such circumstances in a manner similar to that seen in chloramphenicol-treated cells.

Eagle and Saz (8) believed that the inhibition of protein synthesis observed by Gale and Folkes (4) was caused by a primary inhibition of a nitro reductase system. Colaizzi *et al.* (9) summarized the evidence for this theory which suggested that the site of inhibition was at the level of metalloflavoenzymes. Kremery and Kellen (10) proposed that this property may be more directly related to the development of resistance, although Franklin and Godfrey (11) disagreed with their proposal. Snell and Cheng (12) indicated that several modes of action may exist; Jones and Morrisson

Table II—First-Order Generation-Rate Constants, sec.$^{-1}$ × 10^4, for *E. coli* W Cultures Inhibited by Tetracyclines, $M \times 10^6$, and the Fraction of Viable to Total Cells Present at the Start, ϕ_1, and at the End, ϕ_5, of the Experiments

M	k_v	k_t[a]	k_p	$k_{n.a.}$	ϕ_1[b]	ϕ_5[b]
7-Amino-dm-do-tetracycline[c]						
0.000	5.84	5.87	5.46	5.93	0.84	0.81
0.493	4.93	4.80	4.32	4.72	0.80	0.96
0.985	3.71	3.83	3.36	3.66	0.75	0.63
1.478	2.98	3.05	2.74	2.77	0.68	0.62
1.971	2.41	2.41	1.96	2.02	0.72	0.71
9-Amino-dm-do-tetracycline						
0.000	5.84	5.87	5.46	5.93	0.84	0.81
0.474	5.27	5.27	4.61	4.86	0.74	0.74
0.948	4.06	4.59	3.91	4.08	0.87	0.40
1.421	3.79	3.73	3.38	3.44	0.72	0.80
1.895	—	3.06	3.02	3.17	—	—
5*a*(6)-Anhydrotetracycline						
0.000	5.82	5.62	5.40	5.90	0.91	1.21
4.477	5.00	5.46	4.70	4.90	0.69	0.35
8.953	4.38	4.67	3.79	3.87	0.79	0.52
13.430	4.12	3.88	3.25	3.45	0.64	0.90
17.907	—	3.32	2.56	2.77	—	—
7-Nitro-dm-do-tetracycline						
0.000	5.82	5.62	5.40	5.90	0.91	1.21
0.111	5.68	5.50	4.86	5.44	0.67	0.87
0.222	4.20	4.75	3.88	4.32	0.74	0.33
0.333	3.17	3.82	3.11	3.52	0.72	0.28
0.444	2.45	2.82	2.19	2.59	1.00	0.61
9-Nitro-dm-do-tetracycline						
0.000	6.10	5.86	5.81	6.14	0.74	1.05
2.337	4.77	5.37	5.07	5.15	0.97	0.41
4.674	3.93	4.11	3.48	3.54	0.68	0.53
7.011	2.93	2.96	2.82	2.55	0.82	0.77
9.348	1.95	2.02	1.92	1.85	0.69	0.62
4-Dedimethylaminotetracycline						
0.000	6.10	5.86	5.81	6.14	0.74	1.05
3.030	5.27	5.39	4.63	5.01	0.79	0.67
6.060	4.22	4.50	3.96	4.30	0.80	0.53
9.091	3.43	3.80	3.58	3.51	0.86	0.50
12.121	3.17	3.12	2.97	3.04	0.65	0.70
9-Dimethylamino-dm-do-tetracycline						
0.000	6.25	6.09	5.45	5.40	0.66	0.83
3.467	5.60	5.71	5.01	5.00	0.87	0.74
6.935	4.32	4.69	3.92	3.99	0.70	0.41
10.402	3.34	3.47	3.09	3.51	0.60	0.50
13.870	2.83	2.94	2.40	2.56	0.74	0.63
Tetracycline methiodide						
0.00	6.25	6.09	5.45	5.40	0.66	0.83
63.48	4.87	5.14	4.03	4.26	0.83	0.56
126.95	3.02	3.39	2.78	2.91	0.71	0.42

[a] Values of k_t were obtained with 0.2% HCHO in the diluting solution see *Experimental*). [b] ϕ was calculated from Eq. 1 using the values $k_{e.a.}$ and N_0, obtained by regression analysis of the original data. [c] dm-do- is 6-demethyl-6-deoxy-.

(13, 14) and Benbough and Morrisson (15) provided evidence for this view but believed that the primary action is a direct inhibition of protein synthesis.

Franklin (16), in experiments with cell-free extracts designed to prove that an inhibition of protein synthesis was a direct effect of the tetracyclines, showed that protein synthesis is directly inhibited at the level of incorporation of aminoacyl-sRNA. Laskin and Chan (17) reached similar conclusions from studies of synthetic messenger-directed protein-synthesizing extracts. Hierowski (18) and Suarez and Nathans (19) showed that inhibition is caused by preventing the binding of aminoacyl-sRNA to ribosome-mRNA complexes; their experiments were confirmed by other workers (20–23). Lucas-Lenard and Haenni (24) showed that

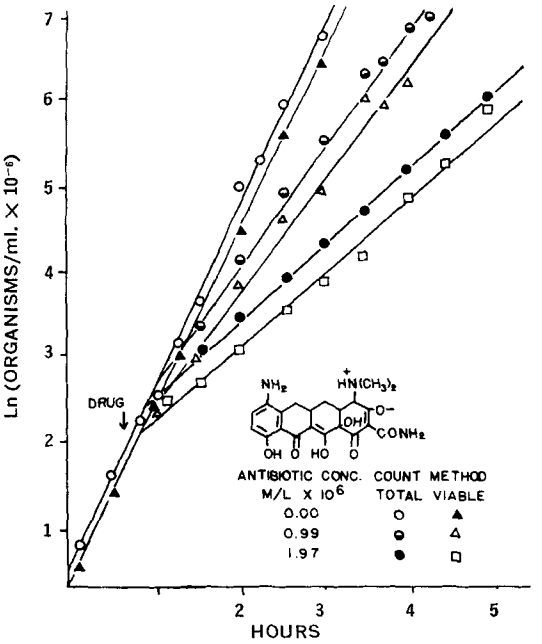

Figure 1—*Generation-rate curves for cell division in the presence of various concentrations of 7-amino-6-demethyl-6-deoxytetracycline. Samples obtained from each culture were counted by a viable and a total count method. The slopes,* k_v *and* k_t, *are given in Table II.*

the GTP-dependent binding of aminoacyl-sRNA at low magnesium-ion concentrations is dependent upon the presence of the T-factor of Nishizuka and Lipmann (25). The effect of chlortetracycline is to prevent binding of aminoacyl-sRNA at a level beyond that involving the formation of an initial aminoacyl-sRNA–GTP–T complex (24). Connamacher and Mandel (20) and Day (26–28) studied the binding of tetracycline to ribosomes and believed that the binding is related to the prevention of aminoacyl-sRNA binding. Maxwell (29–31) investigated this binding as well as other factors and was led to similar conclusions. Cundliffe (32) described tetracycline effects on polyribosomes in growing protoplasts of *Bacillus megaterium* which are consistent with this hypothesized mode of action. Rifino *et al.* (33) studied the effects of several tetracycline analogs on cell-free protein-synthesizing systems in addition to those studied by Laskin and Chan (17) and Hierowski (18), and they reported preliminary correlations of antibacterial activity in synthetic messenger-directed systems but not in those directed by natural messengers.

EXPERIMENTAL

Materials—*E. coli* W, obtained as a frozen paste,[1] was cultured over several growth cycles in broth and on agar plates. An isolate from a single colony was used to inoculate a nutrient agar slant, which was maintained by monthly transfers. The culture broth was a high peptone media containing, in a volume of 1 l., 40 g. casein hydrolysate (GBI No. 20), 15 g. yeast autolysate (GBI No. 120), 3 g. K_2HPO_4, and 0.7 g. KH_2PO_4. The pH was maintained constant at 6.7 throughout the experiments. For experiments at a pH value of 6.2, the broth contained 0.5 g. K_2HPO_4 and 2.5 g. KH_2PO_4, while 21.85 g. of K_2HPO_4 and 1.7 g. of KH_2PO_4 were used in addition to the peptones for those experiments at pH 7.4. The dextrose-salts broth (pH 6.7) used contained, in a volume of 1 l., 10 g. dextrose, 3 g. $(NH_4)_2SO_4$, 0.1 g. $MgSO_4$, 6 g. K_2HPO_4, and 8 g. KH_2PO_4. Broth was filtered through cellulose ester membranes[2] (0.22-μ diameter

[1] General Biochemicals, Chagrin Falls, Ohio.
[2] Millipore Corp., Bedford, Mass.

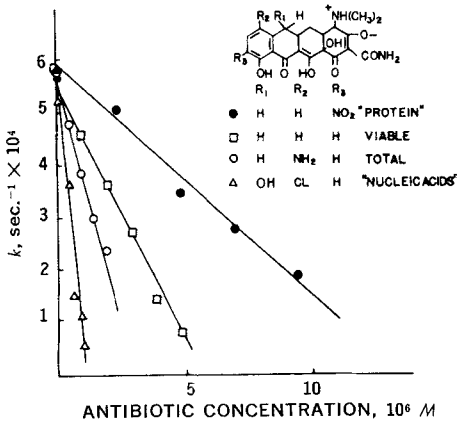

Figure 2—*The linear dependence of generation-rate constants*—k_v, k_t, k_p, *and* $k_{n.a.}$—*upon antibiotic concentration for several different tetracyclines. The lines shown are the average inhibitory rate constants,* $k^1_{avg.}$, *for each compound and are given in Table III.*

pores) and autoclaved prior to use. Samples of various tetracycline analogs were supplied by the manufacturers.[3] The nomenclature used was described previously (9).

Growth—A broth culture was allowed to grow for 12 hr. at 37.5°. A dilution of this culture, whose growth was measured turbidimetrically, was allowed to grow into the logarithmic phase. When a concentration of 1×10^8 organisms/ml. was attained, 2-ml. samples were used to inoculate nine replicate 100-ml. volumes of broth in loosely capped 500-ml. flasks. The flasks were shaken in a constant-temperature water bath (37.5°). Freshly prepared antibiotic solutions were added in 1-ml. volumes to eight of the nine replicate cultures after 2700 sec. of growth. Experiments designed to study time of onset of inhibition were performed with cultures of either 200 ml. /1-l. flask or 350 ml./2-l. flask.

Viable Count Method—One-milliliter samples of the culture were diluted with 0.9% saline solution so that a concentration of 100–200 organisms/ml. would result. One milliliter of this suspension was pipeted into each of five Petri dishes containing solidified peptone–casein agar USP[4] and then covered with melted agar. The plates were incubated for 36–48 hr. at 37.5°, and the colonies were counted (2).

Total Count Method—One-milliliter samples of the cultures were placed in bottles containing appropriate volumes of a saline–

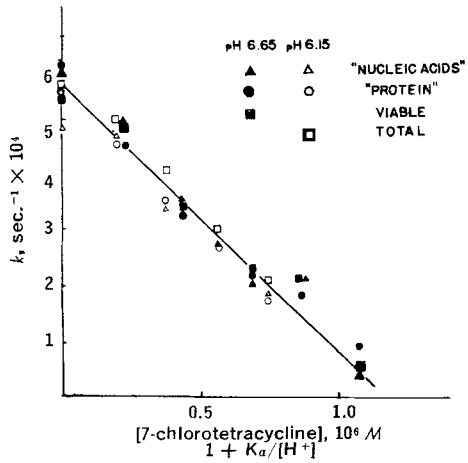

Figure 3—*The linear dependence of generation-rate constants obtained at two broth pH values upon the concentration of the zwitterionic form of 7-chlorotetracycline. The slope is −470 l./mole-sec.*

[3] The authors are especially grateful to Dr. James H. Boothe, Lederle Laboratories, Pearl River, N. Y., and Dr. Charles R. Stephens, Charles Pfizer Laboratories, Groton, Conn., for their assistance in providing the tetracycline analogs employed in this study.

[4] Fisher Scientific, Fairlawn, N. J.

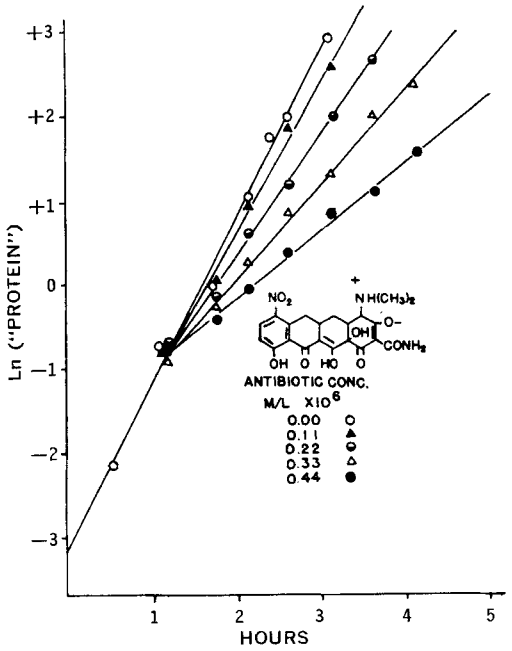

Figure 4—*Generation-rate curves for protein synthesis in the presence of various concentrations of 7-nitro-6-demethyl-6-deoxytetracycline. The slopes,* k_p, *are given in Table II.*

formaldehyde solution so that a concentration of 10,000–30,000 organisms/50 μl. was obtained. This solution had previously been filtered to remove particulate contamination. The HCHO concentration was either 0.2 or 1%; the higher concentration gave values of total cells which remained constant for 24 hr., while the lower concentration allowed some growth (1–5%) in the first 10 min. after samples were obtained. Diluted samples were counted with a Coulter counter model B[6] equipped with a 30-μ orifice (3). Instrument settings that gave satisfactory results were: 1/aperture current, 0.707; 1/amplification, 1/4; matching switch, 32H; gain, 100; lower

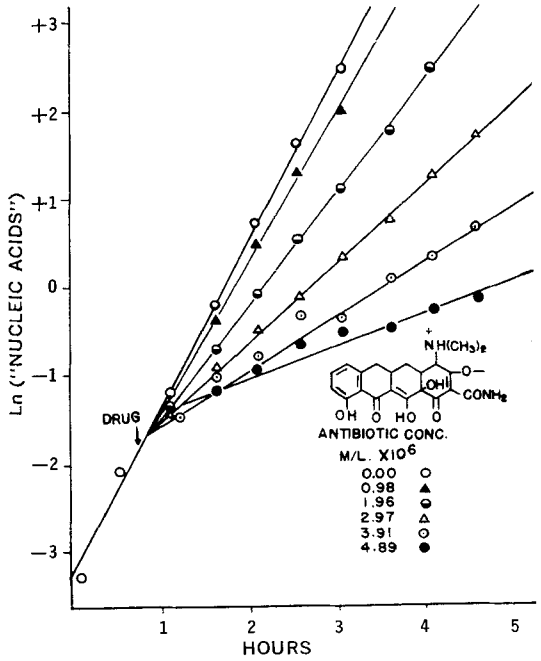

Figure 5—*Generation-rate curves for nucleic acid synthesis in the presence of various concentrations of 6-demethyl-6-deoxytetracycline. The slopes,* $k_{n.a.}$, *are given in Table I.*

[5] Coulter Electronics, Inc., Hialeah, Fla.

Table III—Inhibitory Rate Constants, l./mole-sec., for Inhibition of *E. coli* W by Various Tetracyclines[a]

Tetracycline Analog	$-k_v^I$	$-k_t^I$	$-k_p^I$	$-k_{n.a.}^I$	$-k_{avg.}^I$
7-Nitro-dm-do-tetracycline[b]	833.51	656.76	735.68	769.19	748.78[c]
7-Chloro-dm-tetracycline	591.86	—[d]	460.25	536.48	517.21
7-Chlorotetracycline	387.77	—	396.14	420.20	401.37
Tetracycline	267.84	—	279.97	267.95	271.50
5-Hydroxytetracycline	275.19	—	254.79	251.83	257.15
7-Amino-dm-do-tetracycline	179.01	175.90	174.08	197.95	181.73[c]
9-Amino-dm-do-tetracycline	155.53	151.23	128.82	146.13	145.03[e]
dm-do-Tetracycline	103.62	—	86.70	92.93	94.42[e]
7-Bromo-dm-do-tetracycline	—	62.68	46.09	51.42	51.79
9-Nitro-dm-do-tetracycline	43.40	43.15	42.91	47.86	44.33
4-Dedimethylaminotetracycline	25.39	23.29	22.24	25.45	24.09
9-Dimethylamino-dm-do-tetracycline	26.22	24.62	23.11	20.73	23.67[c]
5*a*(6)-Anhydrotetracycline	12.75	13.81	15.91	17.23	15.48[c]
12*a*-Deoxytetracycline	—	2.63	3.85	2.37	2.62
Tetracycline methiodide	2.54	2.12	1.96	2.10	2.18
5*a*(11*a*)-Dehydrochlortetracycline	—	0.36	0.25	0.42	0.34
7-Chloro-isotetracycline			No activity observed		
2-Cyano-2-decarboxamido-tetracycline			No activity observed		

[a] Tested by analysis of covariance to determine if significant differences existed between the inhibitory rate constant determined using all values of generation-rate constants collectively, $k_{avg.}^I$, and individual inhibitory rate contants determined separately. [b] dm-do- is 6-demethyl-6-deoxy-. [c] Significant at $p > 0.975$. See, for example, R. Steel and J. Torrie, "Principles and Procedures of Statistics," McGraw-Hill, New York, N. Y., 1960, p. 174. [d] Insufficient data available for testing by analysis of covariance. [e] Indicates significant differences at $p > 0.95$.

threshold, 10; and upper threshold, off. The mean of four total counts per sample was obtained and corrected for background noise (200–600 counts).

Nucleic Acid and Protein Determinations—Appropriate volumes of culture (0.5–25 ml.) were filtered under vacuum through 25-mm. diameter cellulose ester membranes[2] (0.22-μ diameter pores), which had previously been treated to extract detergent. Detergent extraction was carried out by placing filters in cold distilled water—4 l./100 filters—and boiling the suspension for 15 min. This process was repeated three times with fresh water (34). Filters treated in this manner gave minimal and constant blank reactions with the orcinol reagent and were able to retain *E. coli* quantitatively. The retained *E. coli* were washed with 0.9% saline solution under vacuum, after which the bacteria were precipitated by layering, without vacuum, 2 ml. of 5% TCA over the filters for 5 min. TCA-soluble material was removed by application of vacuum, and 2 ml. of 95% ethanol was layered over the filters for 5 min. After removal of the ethanolic extract, the filters with retained nucleic acids and proteins were placed in screw-cap test tubes. Two milliliters of 5% TCA was added, and the tubes were placed in a boiling water bath for 20 min.

Nucleic Acids—One-milliliter aliquots of the hot TCA-soluble material were removed from the cooled tubes. Three milliliters of the orcinol reagent (35) was added; after heating in a boiling water bath for 20 min., the absorption at 650 nm. of the cooled solution was measured. This absorption was corrected for that produced when an equal volume of sterile broth instead of culture was applied to the filters. It was necessary to prepare separate blank reactions for each lot of filters and broth. The quantity reported as "nucleic acids" is the corrected absorbance due to a 12.5-ml. culture sample in an orcinol reagent volume of 4 ml.

Proteins—The TCA solution was removed completely by vacuum aspiration from the tubes containing the filters. The filters with retained protein precipitate were covered with 1.5 ml. of a trypsin solution [10 mcg./ml. in 0.05 *M* $(NH_4)_2CO_3$ and 0.005 *M* NH_4OH]. The usual 0.1 *N* alkali protein digestant hydrolyzed the filters and gave rise to high blank values. After the precipitate was mixed vigorously, it was allowed to digest for 2 hr. (up to 48 hr. was not harmful). The resulting solution in the presence of the filters was used as a sample for protein determination by the Folin–Lowry method. The reagents, prepared according to the method of Layne (36), were added directly to the tubes, and the absorbance of the solutions was measured at 750 nm. The resulting absorbance was corrected for that occurring in the blank reactions, and the absorbance due to a 25-ml. sample of culture contained in a final reaction volume of 6.5 ml. is reported as "protein."

The protein and nucleic acid content of *E. coli* cultures collected on cellulose membranes and treated as described in this report was comparable to that observed when bacteria were collected by centrifugation (5). The method reported here appears to be more reproducible and more convenient when handling large numbers of samples.

RESULTS AND DISCUSSION

Effect of Tetracyclines on Rate Constants for Cell Division—It was previously shown (3) that the effect on the culture population of tetracycline at low concentrations is a reduction in the generation-rate constant; that is:

$$N = N_0 e^{k_{c.d.}t} \qquad k_0 < k_{c.d.} > 0 \qquad \text{(Eq. 1)}$$

where N is the concentration of cells, $k_{c.d.}$ is the generation-rate constant for cell division in the presence of antibiotic, k_0 is the rate constant in the absence of antibiotic, and t is the time. The present results show that Eq. 1 satisfactorily describes the equilibrium inhibition of *E. coli* W cultures produced by low concentrations of 16 tetracycline derivatives. F tests for the fit of experimental data to Eq. 1 were found to be highly significant in cases where data obtained for times greater than 30 min. but less than 4 hr. after antibiotic addition were used. Rate constants obtained in this way are given in Tables I and II. Two experimental methods were used to obtain $k_{c.d.}$. The constant k_v is from viable plate counts, and the constant k_t is from total cell counts obtained with the Coulter counter. Rate constants obtained by the two methods were identical within experimental error (Table II). However, in *E. coli* W cultures—in contrast to previous work with *E. coli* B/r (3)—the concentrations of cells at any time, t, as determined by the two methods, were quite different (Fig. 1). It is, therefore, not possible in the present work to conclude with certainty that the antibiotics act solely as growth inhibitors. However, the fraction of viable cells to total cells, φ, remained relatively constant in most cultures before and after addition of antibiotics (Table II). In the cases where it did vary, it did not appear to be consistently related to antibiotic concentration. At higher antibiotic concentrations, this fraction, ϕ, had values of 0.1 or 0.01. The authors believe that there is no large antibiotic-induced death of cells, at these concentrations, but feel that a constant fraction of colonies formed on plates is the result of more than one viable cell. Inclusion of a surfactant in the diluting solution did not, however, greatly affect this fraction. The use of a higher HCHO concentration in the diluting fluid for total counts did make this fraction more constant.

Rate constants for cell division were found to obey the simple equation:

$$k_{c.d.} = k_0 - k_{c.d.}^I \{Tc\} \qquad \text{iff } k_{c.d.} > 0 \qquad \text{(Eq. 2)}$$

where $k_{c.d.}$ may be either k_v or k_t and $\{Tc\}$ is the antibiotic concentration. Figures 2 and 3 are examples of the fit of experimental data to this equation. The experimentally observed rate constants do not

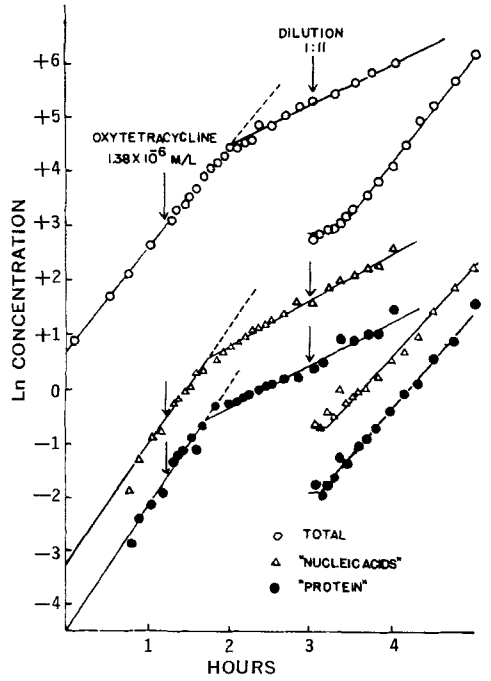

Figure 6—*Generation-rate curves for cell division, by the total count method, nucleic acid synthesis, and protein synthesis in the presence of 5-hydroxytetracycline and a 1:11 dilution of the inhibited culture. The generation-rate curve for protein synthesis has been transposed by subtracting 0.5 from the actual value of* Ln_e *"protein." The generation-rate constants, sec.*$^{-1} \times 10^4$*, for the undiluted culture are given in Table I, except for culture turbidity (not shown) which had values of 5.63 and 2.19 in the absence and presence of antibiotic, respectively. The values in the diluted culture were:* $k_t = 5.43$, $k_p = 5.09$, $k_{n.a.} = 4.61$, k_v *(not shown)* $= 5.33$ *and for turbidity 4.76. The time of antibiotic addition was 4470 sec., and the time of dilution was 10,850 sec. The times of onset of inhibition and the times of onset of recovery calculated as the intersection of the regression lines, respectively, in seconds were: for protein synthesis, 6200 and 11,800; for turbidity (not shown), 6400 and 11,500; for nucleic acid synthesis, 6400 and 12,400; and for cell division—total, 7100 and 12,500, and viable (not shown), 6500 and 12,200.*

fit the more complete expression:

$$\frac{1}{k_0 - k_{c.d.}} = k^I\,(1/\{Tc\} + k) \qquad \text{(Eq. 3)}$$

where k^I and k are constants. Equation 3 was derived by Garrett *et al.* (37).

Effect of Tetracyclines on Rate Constants for Protein and Nucleic Acid Syntheses—As noted, many workers (4–7) reported inhibition of protein synthesis at low tetracycline concentrations. Many of these workers also reported different effects on nucleic acid synthesis, either a stimulation (4, 5) or a lag time before onset of inhibition (6, 7). The present work shows that an equation similar to Eq. 1, which described the concentration of cells, can be written for both protein and nucleic acid concentrations (Figs. 4 and 5). Values of $k_{n.a.}$ and k_p derived from this equation are given in Tables I and II. In addition, these generation-rate constants are related to antibiotic concentration in a manner similar to the relationship of cell division to antibiotic:

$$k_p = k_0 - k_p{}^I\,\{Tc\} \qquad \text{(Eq. 4)}$$

$$k_{n.a.} = k_0 - k_{n.a.}{}^I\,\{Tc\} \qquad \text{iff } k_{c.d.} > 0 \qquad \text{(Eq. 5)}$$

Values of the inhibitory rate constants, $k_v{}^I$, $k_t{}^I$, $k_p{}^I$, and $k_{n.a.}{}^I$, obtained for 18 antibiotics are listed in Table III. The hypothesis was tested that these values are experimentally indistinguishable, and no statistically significant differences were found between the individual values and an average for seven of these antibiotics. Statistically significant differences were found for the six other antibiotics tested (Table III). This simple test did not consider the error involved in the determination of the individual generation-rate constants. The authors believe that within experimental accuracy, the four parameters are the same for all antibiotics. That is, a one-to-one relationship is found among these parameters not only in control cultures but also in antibiotic-inhibited cultures. The inhibited cultures are growing in balanced growth, and the rate of cell division is proportional to the total rate of protein synthesis rather than to a net rate as was previously postulated (37). The net rate theory assumed that a minimum rate of protein synthesis was necessary to maintain viability. Additional protein synthesis was needed for cell division and, it was believed, the tetracycline selectively inhibited the protein synthesis needed for cell division.

Time of Onset of Inhibiton—The fact that inhibited cultures exhibit parallel rates of cell division and protein and nucleic acid syntheses is not in accord with the interpretation of previous experiments (4, 5). These experiments were single-point measurements and noted decreased protein/cell and increased nucleic acid content/cell. However, Cerny and Habermann (6), in a kinetic experiment, showed parallel rates of protein and nucleic acid syntheses with different times of onset of action. Their work was done at antibiotic concentrations 15 times higher than those presently employed, which caused complete inhibition and undoubtedly cell death. Experiments of the type shown in Figs. 1, 4, and 5 gave indications that at these low concentrations the effect of antibiotics was also not immediate.

Figure 6 shows an experiment designed to document this observation. It can easily be seen that the onset of inhibition for all parameters is considerably beyond the time of antibiotic addition. Viable plate counts (not shown) paralleled those obtained by the Coulter counter. This experiment was repeated at a different oxytetracycline concentration with similar results. In addition, Fig. 6 shows that the time of onset of recovery from inhibition upon dilution of the

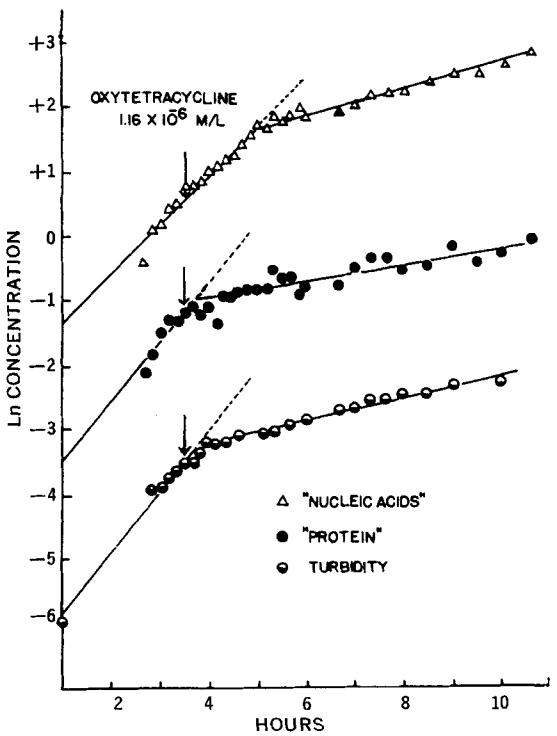

Figure 7—*Generation-rate curves in a dextrose–salts broth for nucleic acid synthesis, protein synthesis, and culture turbidity in the presence of 5-hydroxytetracycline. The generation-rate curve for nucleic acid synthesis has been transposed by adding 2.0 to the actual value of* Ln_e *"nucleic acids." Values of generation-rate constants, sec.*$^{-1} \times 10^4$*, in the absence and presence of antibiotic, respectively, were:* $k_{n.a.} = 2.13$ *and 0.56,* $k_p = 2.62$ *and 0.37,* k_t *(not shown)* $= 2.44$ *and 0.12 and for turbidity 2.54 and 0.37. The time of antibiotic addition was 8900 sec. The times of onset of inhibition, in seconds, calculated as the intersection of the regression lines were: for protein synthesis, 9900; for turbidity, 10,300; for cell division, 10,750; and for nucleic acid synthesis, 14,200.*

Table IV—First-Order Generation-Rate Constants, sec.$^{-1}$ × 10^4, and Inhibitory Rate Constants, l./mole-sec., for *E. coli* W in Various Broth Cultures Inhibited by Several Tetracyclines

10^6 *M*	k_t	k_p	$k_{n.a.}$	$k^I_{avg.}$
7-Chlorotetracycline, peptone broth, pH 6.2				
0.000	5.56	5.50	4.88	
0.192	4.95	4.55	4.66	
0.384	4.06	3.50	3.38	
0.575	3.01	2.69	2.71	
0.767	2.10	1.78	1.86	
k^I	−461.11	−484.15	−417.52	−454.28[a]
5a(6)-Anhydrotetracycline, peptone broth, pH 6.2				
0.00	5.56	5.50	4.88	
4.67	4.71	4.68	3.90	
9.34	3.94	3.23	3.45	
14.01	3.35	2.57	2.92	
18.68	1.73	1.99	2.35	
k^I	−19.27	−19.54	−12.95	−17.25[a]
4-Dimethylaminotetracycline, peptone broth, pH 7.4				
0.00	4.57	5.22	4.76	
3.34	3.87	5.01	3.96	
6.65	3.33	3.73	3.48	
9.96	2.71	3.03	3.00	
13.24	2.09	2.13	2.54	
k^I	−18.48	−24.70	−16.32	−19.83[a]
7-Chlorotetracycline, pH 6.7 dextrose–salts broth				
0.000	2.39	2.30	2.18	
0.163	2.07	1.92	2.01	
0.327	0.97	0.84	0.79	
0.490	0.39	0.51	0.50	
0.655	0.22	0.38	0.37	
k^I	−368.34	−315.81	−312.59	−332.25

[a] Significant differences between inhibitory rate constants determined by analysis of covariance as explained in Table III.

cultures into antibiotic free media is not immediate either but also exhibits a lag time.

Inhibition and recovery of protein synthesis seemed to occur slightly before the other parameters were affected. To test this observation, an experiment was performed in a dextrose–salts broth where the rate of cell division in control cultures was half that in the peptone broth. This single experiment (Fig. 7) indicates that protein synthesis measured by the Folin–Lowry technique and cell mass measured by turbidity are most likely inhibited sooner than nucleic acid synthesis. Cell division (not shown) was inhibited at an intermediate time. The small number of data points obtained in this experiment before the addition of antibiotic, necessitated by the culture conditions, makes it difficult to calculate the times of onset of inhibition with a high degree of confidence. However, it seems most likely that the rates of increase for all parameters in the absence of antibiotic are the same in this broth, just as they were in the peptone broth (38). Therefore, the conclusion of different times of onset of inhibition calculated from this experiment (Fig. 7) seems valid.

The existence of different growth rate-dependent lag times, as observed in this experiment, would explain the discrepancy between parallel equilibrium inhibition rates and nonparallel amounts of protein and nucleic acid per cell. This order of inhibition and recovery is consistent with the hypothesized primary inhibition of protein synthesis followed by a secondary, indirect inhibition of nucleic acid synthesis and cell division due to a lack of protein. In addition to the growth rate-dependent difference in time of onset of inhibition of the various culture parameters, a lag time is observed before any parameter is inhibited (Fig. 7). Two general explanations are possible for this lag time. First, a finite time may be required to establish equilibrium concentrations within the cells. Second, antibiotic may permeate the cell and arrive at the receptor site but require finite time to exert a measurable effect. Since this lag time is not growth-rate dependent and does vary with broth conditions, which do not change the growth rate, the first explanation is preferred.

Effect of Broth pH Changes on Inhibition by Tetracyclines—Jones and Morrisson (13) and Benbough and Morrisson (15) reported that when the pH of culture media of known free metal-ion concentration is varied, the inhibition produced is proportional only to the "molecular," presumably zwitterionic, species of several tetracyclines. Because of the slow growth rate characteristics of *E. coli* W in dextrose–salts broth, it was not possible to verify these results for other tetracyclines. However, experiments in peptone broth where the free metal-ion concentration is not precisely known are consistent with this result.[6] Figure 3 shows a plot of Eq. 2 for experiments at two pH values, where the concentration of antibiotic is corrected so that it reflects the concentration of zwitterionic species present, assuming the fraction of total tetracycline chelated to metal ions of the medium is the same at both pH values. Activities obtained at a broth pH of 7.4, where a much lower free metal-ion concentration would be expected, do not fit the single line plotted in Fig. 3. Results obtained with 5a(6)-anhydrotetracycline (pH 6.2 and 6.7) and 4-dedimethylaminotetracycline (pH 6.7 and 7.4) (Table IV) do not fit such a simple concept either. Connamacher *et al.* (39) recently reported data on uptake of tetracycline in *Bacillus cereus* cultures at pH 6.7 and 7.3 and at two magnesium-ion concentrations which are in qualitative agreement with the hypothesis of Jones and Morrisson (13, 14).

In addition to causing a change in the degree of equilibrium inhibition produced by a given total antibiotic concentration, pH changes are also capable of changing the observed lag time for onset of inhibition. Figure 8 shows an experiment identical to that presented previously (Fig. 6), except that the pH of the broth was 7.4 instead of 6.7. The lag for onset of inhibition of protein synthesis (measured as cell mass) and cell division is seen to be shortened by two-thirds. The equilibrium amount of inhibition achieved was the same in both experiments. If the hypothesis of Jones and Morrisson (13, 14) is correct, then the concentration of zwitterionic species

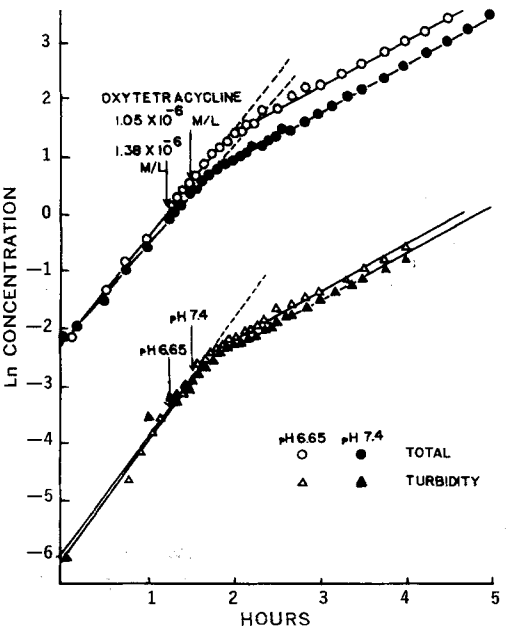

Figure 8—*Generation-rate curves, done on separate days at two peptone broth pH values, for cell division by the total count method and culture turbidity in the presence of 5-hydroxytetracycline. The generation-rate curves for cell division have been transposed by subtracting 3.0 from the actual value of* Ln_e *organisms/ml. The generation-rate constants, sec.*$^{-1}$ × 10^4, *in the absence and presence of antibiotic, respectively, for cell division were 5.16 and 2.20 at pH 6.7 and 4.75 and 2.30 at pH 7.4, and for turbidity were 5.63 and 2.19 at pH 6.7 and 5.56 and 2.21 at pH 7.4. The times of onset of inhibition, in seconds, calculated as the intersection of the regression lines were: for cell division, 7100 and 6200; and for turbidity, 6400 and 5900 at pH 6.7 and 7.4, respectively.*

[6] The divalent metal-ion concentration, expressed as calcium, in the broths has been estimated with a divalent metal-ion electrode to be approximately 10^{-4} *M* (Orion Research Inc., Cambridge, MA 02139). The calcium-ion concentration in these broths has been determined by Dr. Bartholomew van't-Riet, Department of Chemistry, Medical College of Virginia (titration with ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid) to be 1.0 to 1.1 × 10^{-4} *M*.

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.

REFERENCES

(1) G. C. Barrett, *J. Pharm. Sci.*, **52,** 309(1963).
(2) M. R. W. Brown and E. R. Garrett, *ibid.*, **53,** 179(1964).
(3) E. R. Garrett and G. H. Miller, *ibid.*, **54,** 427(1965).
(4) E. F. Gale and J. P. Folkes, *Biochem. J.*, **53,** 493(1953).
(5) J. H. Hash, M. Wishnick, and P. A. Miller, *J. Biol. Chem.*, **239,** 2070(1964).
(6) R. Cerny and V. Habermann, *Collect. Czech. Chem. Commun.*, **29,** 1326(1964).
(7) I. A. Holmes and D. G. Wild, *Biochem. J.*, **97,** 277(1965).
(8) H. Eagle and A. K. Saz, *Ann. Rev. Microbiol.*, **9,** 173(1955).
(9) J. L. Colaizzi, A. M. Knevel, and A. N. Martin, *J. Pharm. Sci.*, **54,** 1425(1965).
(10) V. Krcmery and J. Kellen, *J. Bacteriol.*, **92,** 1264(1966).
(11) T. J. Franklin and A. Godfrey, *Biochem. J.*, **94,** 54(1965).
(12) J. F. Snell and L. Cheng, *Dev. Ind. Microbiol.*, **2,** 107(1961).
(13) J. G. Jones and G. A. Morrisson, *J. Pharm. Pharmacol.*, **14,** 808(1962).
(14) *Ibid.*, **15,** 34(1963).
(15) J. Benbough and G. A. Morrisson, *J. Pharm. Pharmacol.*, **17,** 409(1965).
(16) T. J. Franklin, *Biochem. J.*, **87,** 449(1963).
(17) A. I. Laskin and W. M. Chan, *Biochem. Biophys. Res. Commun.*, **14,** 137(1964).
(18) M. Hierowski, *Proc. Nat. Acad. Sci. USA*, **53,** 594(1965).
(19) G. Suarez and D. Nathans, *Biochem. Biophys. Res. Commun.*, **18,** 743(1965).
(20) R. H. Connamacher and H. G. Mandel, *ibid.*, **20,** 98(1965).
(21) J. M. Clark, Jr., and A. Y. Chang, *J. Biol. Chem.*, **240,** 4734 (1965).
(22) I. Suzuka, H. Kaji, and A. Kaji, *Proc. Nat. Acad. Sci. USA*, **55,** 1483(1966).
(23) D. Vazquez and R. E. Monro, *Biochim. Biophys. Acta*, **142,** 155(1967).
(24) J. Lucas-Lenard and A. Haenni, *Proc. Nat. Acad. Sci. USA*, **59,** 544(1968).
(25) Y. Nishizuka and F. Lipmann, *ibid.*, **55,** 212(1966).
(26) L. E. Day, *J. Bacteriol.*, **91,** 1917(1966).
(27) *Ibid.*, **92,** 197(1966).
(28) *Ibid.*, **92,** 1263(1966).
(29) I. H. Maxwell, *Biochim. Biophys. Acta*, **138,** 329(1967).
(30) *Ibid.*, **138,** 337(1967).
(31) I. H. Maxwell, *Mol. Pharmacol.*, **4,** 25(1968).
(32) E. Cundliffe, *ibid.*, **3,** 401(1967).
(33) C. V. Rifino, W. F. Bousquet, A. M. Knevel, and A. N. Martin, *J. Pharm. Sci.*, **57,** 351(1968).
(34) R. D. Cahn, *Science*, **155,** 195(1967).
(35) W. C. Schneider, in "Methods of Enzymology," vol. 3, S. P. Colowick and N. O. Kaplan, Eds., Academic, New York, N. Y., 1957, p. 680.
(36) E. Layne, *ibid.*, p. 447.
(37) E. R. Garrett, G. H. Miller, and M. R. W. Brown, *J. Pharm. Sci.*, **55,** 593(1966).
(38) O. Maaloe and N. O. Kjeldgaard, "Control of Macromolecular Synthesis," W. A. Benjamin, New York, N. Y., 1966, p. 90.
(39) R. H. Connamacher, H. G. Mandel, and F. E. Hahn, *Mol. Pharmacol.*, **3,** 586(1967).
(40) K. Arima and K. Izaki, *Nature*, **200,** 192(1963).
(41) K. Izaki and K. Arima, *J. Bacteriol.*, **89,** 1335(1965).
(42) K. Izaki, K. Kiuchi, and K. Arima, *ibid.*, **91,** 628(1966).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 22, 1968, from the *School of Pharmacy, Medical College of Virginia, Richmond, VA 23219*

Accepted for publication July 21, 1970.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

The authors thank Luisa C. Miller and Sung Ho Hahn for their technical assistance. The authors thank the members of the Department of Biometry, Medical College of Virginia, for their help.

* Present address: School of Pharmacy, Medical College of Virginia, Richmond, VA 23219

† Present address: Faculty of Pharmacy, Alexandria University, Alexandria, Egypt, UAR.

‡ Present address: Temple University School of Pharmacy, Philadelphia, PA 19140; where requests should be directed.

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