α-Ethyl-β-(2,4-diiodo-5-hydroxyphenyl)-propionic Acid.—Four-tenths of a mole of α-ethyl-β-(*m*-hydroxyphenyl)-propionic acid was iodinated with potassium triiodide as described for the α-phenyl compound. The gummy iodinated product from the bisulfite acidification was purified by precipitating the disodium salt from a sodium hydroxide solution with sodium chloride. The disodium salt, after solution in water and acidification, yielded a mixture of a yellow solid and an intractable oil. The solid was recrystallized from carbon tetrachloride and gave 21 g. of the diiodo acid, m.p. 115–116°.

Anal. Calcd. for $C_{11}H_{12}O_3I_2$: C, 29.14; H, 2.99. Found: C, 29.22; H, 2.71.

α-Ethyl-β-(2,4,6-triiodophenyl)-propionic Acid.—To a vigorously agitated solution of 5.7 g. of α-ethyl-β-(3-amino-2,4,6-triiodophenyl)-propionic acid in 50 cc. of concentrated sulfuric acid cooled to 0°, there was added 0.75 g. of finely powdered sodium nitrite. After an additional two hours at 0°, the reaction mixture was poured on approximately 100 g. of ice, the temperature being kept below 5°. The bright yellow slurry which formed was gradually added to a cooled, vigorously stirred suspension of 2.8 g. of cuprous oxide in 210 cc. of 95% ethanol. When the initial evolution of nitrogen had subsided, the mixture was refluxed for about 0.5 hour, at which time no further nitrogen was evolved. The ethanol suspension was diluted with an equal volume of water, kept at room temperature overnight and filtered. The triiodo compound was isolated by ether extraction of the precipitate, the ethereal solution washed with sodium thiosulfate solution, water, dried and evaporated, yield 5 g. Recrystallized from benzene–hexane for analysis, m.p. 151–152.5°.

Anal. Calcd. for $C_{11}H_{11}O_2I_3$: I, 68.5. Found: I, 68.7.

α-Ethyl-β-(2,3,4,6-tetraiodophenyl)-propionic Acid.—α-Ethyl-β-(2,4,6-triiodo-3-aminophenyl)-propionic acid (5.7 g.) in 30 cc. of concentrated sulfuric acid was diazotized with 2.1 g. of sodium nitrite. A solution of 12.7 g. of potassium iodide in 28 cc. of water was added to the cold aqueous yellow slurry of the diazonium salt; and, after the initial vigorous reaction had subsided, the mixture was heated on the steam-bath for one hour. It was then poured into a cold sodium bisulfite solution and the crude tetraiodo acid filtered; yield 7 g., m.p. 150–153°; recrystallized for analysis from acetone–water, m.p. 164–165°.

Anal. Calcd. for $C_{11}H_{10}O_2I_4$: I, 74.5. Found: I, 74.8.

Acknowledgment.—We wish to thank Dr. S. Margolin of our Pharmacology Laboratory for the animal data on the compounds and Mr. E. Conner of our Microanalytical Laboratory for the analyses reported herein.

BLOOMFIELD, NEW JERSEY

[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

Studies on the Structure of Nucleic Acids. VI. The Kinetics of Desoxyribonuclease Action[1]

BY LIEBE F. CAVALIERI AND BARBARA HATCH

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The kinetics of desoxyribonuclease action have been investigated by measuring the liberation of hydrogen ions. This was accomplished by observing the decrease in extinction of the *p*-nitrophenol–phenolate buffer system used. A plot of initial rate *vs.* initial substrate concentration exhibits a maximum which is attributed to inhibition by substrate. Since the order of reaction with respect to time is greater than that with respect to concentration, inhibition by the products of reaction is indicated. This was demonstrated to be the case experimentally. Since the inhibition by products is pronounced, it is suggested that the first products of reaction are nucleic acid-like in nature, rather than small entities. An analysis is set forth which suggests that both the inhibition by substrate and that by products involves the doubly charged phosphate anion though structural features must also be considered.

A study of the kinetics of desoxyribonuclease (DNAase) action is hampered by the fact that the products of reaction are complex entities and do not readily lend themselves to analytical procedures. Changes in viscosity, ultraviolet absorption spectrum and acid precipitability[2] are useful but inadequate since the nature of the linkages involved in these changes is not clearly understood. The measurement of the hydrogen ions produced,[2] which may be used to calculate the number of sugar–phosphate bonds cleaved, appeared to us to be the most direct and feasible route, and one which might ultimately be susceptible to interpretation in terms of the various types of bonds. In the present paper, we describe a simple colorimetric technique which may be used to measure the liberation of acid at a sensibly constant *p*H.

Experimental

Materials.—Sodium desoxyribonucleate was isolated from calf thymus, according to the procedure of Schwander and Signer[3]; $E^{1\%}_{1\ cm.}$ (water), 197. *Anal.* N, 12.7; P, 8.3. Crystalline beef pancreatic desoxyribonuclease was purchased from the Worthington Biochemical Laboratory and was used without further purification. Laboratory distilled water was redistilled from an all-glass apparatus.

Method.—The extent of DNAase action was determined by measuring the quantity of acid liberated. This was achieved by observing the change in optical density of a *p*-nitrophenol–phenolate buffer system containing the enzyme, substrate and magnesium sulfate. To relate optical density with the amount of acid produced, a standard curve was constructed by adding known increments of hydrochloric acid to the buffer system. Under the conditions of the experiments a decrease of one optical density unit at 440 mμ was brought about by the addition of 6.8×10^{-4} equivalent of acid per liter. The solvent cell contained all components except the enzyme. During enzymatic hydrolysis, the system changes by virtue of the fact that the concentration of DNA decreases. To show that this decrease in substrate concentration did not alter the standard curve, various standard curves were constructed in which the DNA was varied down to zero concentration. All curves were found to be identical.

The initial concentration of *p*-nitrophenol was 1×10^{-3} *M* in all cases. The *p*H of the reaction mixture was about 7.1. Since the pK_a of *p*-nitrophenol is 7.16, the phenol was approximately 50% neutralized and therefore at maximum buffer capacity. In general, the initial rates were calculated using values for the concentrations of DNA corre-

(1) This investigation was supported by grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and from the Atomic Energy Commission, Contract AT(30-1)-910.

(2) M. Kunitz, *J. Gen. Physiol.*, **33**, 349 (1949).

(3) H. Schwander and R. Signer, *Helv. Chim. Acta*, **33**, 1521 (1950).

sponding to an increase of less than 0.6×10^{-4} equivalent per liter of acid liberated, *i.e.*, the 2 to 6 minute interval. Therefore, the maximum decrease in *p*H was 0.1 *p*H unit. For the higher substrate concentrations during any one particular time-run, observations are not recorded beyond 2×10^{-4} equivalent of acid per liter, since this corresponds to a decrease of 0.37 *p*H unit. This value represents the largest *p*H change tolerated. Generally, the *p*H variation was from 7.2 (initially) to 7.0 (finally). Though this is a wide range, it was found experimentally that varying the *p*H within these limits did not perceptibly alter the initial rates. It may be concluded, therefore, that in the time-runs in which 2×10^{-4} equivalent was eventually liberated, the observed rates are not seriously in error. In any case, the calculated initial rates are not subject to this type of error, since the *p*H is very nearly constant during the initial phase of reaction. It was ascertained that about one-quarter of the total phosphate of DNA is hydrolyzable[2] by DNAase. The molar concentration of hydrolyzable phosphate was varied between 1×10^{-4} and 10×10^{-4} *M* (0.15–1.50 mg. DNA per ml.).

A second pertinent question is the relative competition between the phenolate ion and the newly-formed secondary phosphoryl group for the liberated hydrogen ion. The pK_a of the secondary phosphoryl dissociation is 6.[4] Thus at *p*H 7 over 90% of this group exists as the anion. Further, since in the extreme case the phenolate ion concentration was over twice as large as the liberated phosphate ion, the amount of acid taken up by the phosphate ion is about 3–4% of the acid liberated. This error applies only to the end of the time-runs at the high substrate concentrations. In all calculated initial rates the error due to this competition is small.

Procedure.—Sodium desoxyribonucleate was made up in a stock solution of about 1.6 mg. per ml. and kept for no longer than 4 days. It was stored at 0°. A buffer stock solution (*p*H 7.2) was prepared which was 0.3 *M* in $MgSO_4$ and 0.01 *M* in *p*-nitrophenol. One ml. of buffer and the appropriate amounts of DNA solution and water comprised the substrate solution. The enzyme concentration was such that when 1 ml. was diluted to 10 ml. (the final volume of the reaction mixture) the resulting concentration was either 3 or 6×10^{-8} *M* (based on a molecular weight of 63,000.[2]) This range of enzyme concentration was chosen so that the observed extent of reaction would take place in about 30 minutes. This was done in order to reduce enzyme inactivation during the reaction to a minimum. This point was checked experimentally and it was found that no perceptible inactivation occurred during enzymatic hydrolysis.

For a run, the substrate mixture and the enzyme solution were brought to constant temperature in a bath at 29.59 ± 0.05°. The enzyme was added rapidly from a calibrated syringe, and the mixture poured into a cuvette and placed in the Beckman spectrophotometer. The temperature during the transfer was 29.5 ± 0.5°, but during the major part of the reaction it remained at 29.5 ± 0.2°. In order to ensure a uniform temperature in the cell chamber, water maintained at a constant temperature was circulated through the metal housing provided for this purpose (National Technical Laboratories, South Pasadena, California).

Some difficulty was encountered with enzyme aging but it was controlled by dissolving the DNAase in the cold at a concentration 50-fold greater than that used in the runs, and stored at −20° for no more than several days. The stock solution was diluted in the cold between 4 and 9 minutes before addition to the substrate. In duplicate runs the calculated initial rates were reproducible to within about 4%, but if different enzyme solutions of various ages were used, the error was two to three times as large.

The concentration of magnesium sulfate in the reaction mixtures was 0.03 *M* in all cases. This concentration is above the optimum amount for enzymatic activity[2] and was sufficiently high to maintain the system at a constant ionic strength.

The possibility that *p*-nitrophenol affects the rate of the reaction was examined by determining the alkaline titer at various times in a system containing no buffer. The measurements were carried out at the glass electrode and general agreement was achieved, although the precision of the latter technique was low. It may be concluded that qualitatively at least, *p*-nitrophenol does not appreciably alter the rate of reaction.

(4) J. M. Gulland, D. O. Jordan and H. F. W. Taylor, *J. Chem. Soc.*, 1131 (1947).

Results

The liberation of acid was followed by observing the change in optical density of the *p*-nitrophenol buffer. The initial optical density was obtained by extrapolation of an optical density *vs.* time plot to zero time. Since readings were generally begun within about 40 seconds, negligible error was introduced by this procedure. From the standard curve described in the Experimental section a plot of substrate concentration, *S*, *vs.* time was readily obtained. About five rates were calculated by the tangent method from the 2 to 6 minute interval. To estimate the initial rate, v_0, a plot of log rate *vs.* log *S* was extrapolated to log S_0. Throughout this paper the concentration of DNA, *S*, is taken as one-quarter of the total phosphate (expressed in terms of potential hydrogen ions), since this is the amount hydrolyzable by DNAase. The variation of initial rate, v_0, with initial substrate concentration, S_0, is shown in Fig. 1 and in the results contained in Table I.

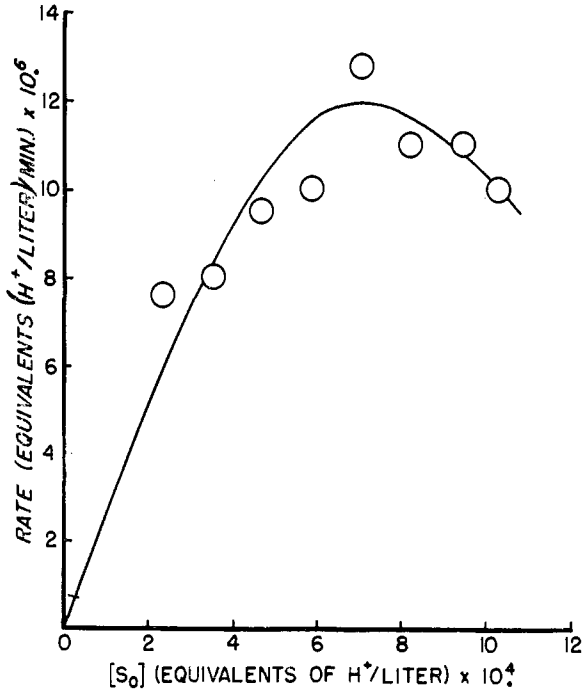

Fig. 1.—The circles represent the experimental values; the solid line is the theoretical curve.

TABLE I

INITIAL RATES AS A FUNCTION OF SUBSTRATE CONCENTRATION

*p*H 7.15 ± 0.15; temp. 29.5 ± 0.2°

$S_0 \times 10^4$ (Equiv. H per liter) {DNAase} →	$v_0 \times 10^6$ 3×10^{-8}	6×10^{-8}	1.2×10^{-8}	15×10^{-8}
2.34	7.6	9.4		
3.51	8.0	10.3		
4.68	9.5	17.3		
5.85	10.0	21.5		
7.00	12.8	23.0		
8.18	11.0	22.0		
9.35	11.0	11.5	4.5	47
10.25	10.0			

ble I. A maximum is apparent at both enzyme concentrations. The method used for calculating the theoretical curve will be taken up in the Discussion section.

TABLE II

SUBSTRATE CONCENTRATION AS A FUNCTION OF TIME

*p*H 7.15 ± 0.15; temp. 29.5 ± 0.2°

S_0 = 2.34 × 10⁻⁴[a]		S_0 = 7.00 × 10⁻⁴		S_0 = 9.35 × 10⁻⁴	
t(min.)	$S \times 10^4$[a]	*t*	$S \times 10^4$	*t*	$S \times 10^4$
0	2.34	0	7.00	0	9.35
1.0	2.23	1.2	6.81	1.2	9.20
1.9	2.17	2.6	6.64	3.0	9.01
4.1	2.04	5.5	6.36	6.7	8.67
6.5	1.95	9.7	6.04	10.0	8.47
8.8	1.89	17.8	5.56	11.7	8.32
12.4	1.79	23.6	5.23	17.4	7.95
18.6	1.69	33.0	4.84	24.6	7.60
25.9	1.60	49.2	4.39	32.5	7.22
38.7	1.51			51.0	6.55
43.0	1.49			70.0	6.14

[a] S_0 and S are expressed in terms of equivalents of hydrolyzable phosphorus per liter.

A plot of S *vs.* time is shown in Fig. 2. The points represented by the various circles are the experimental results. The unbroken curves are calculated theoretically for a competitive inhibition by the products of reaction, while the broken curves are calculated for an uninhibited reaction. In Fig. 3 the ratio v_0/v (uninhibited rate/observed rate) is plotted *vs.* I, the inhibitor concentration, which is taken to be proportional to $(S_0 - S)$.

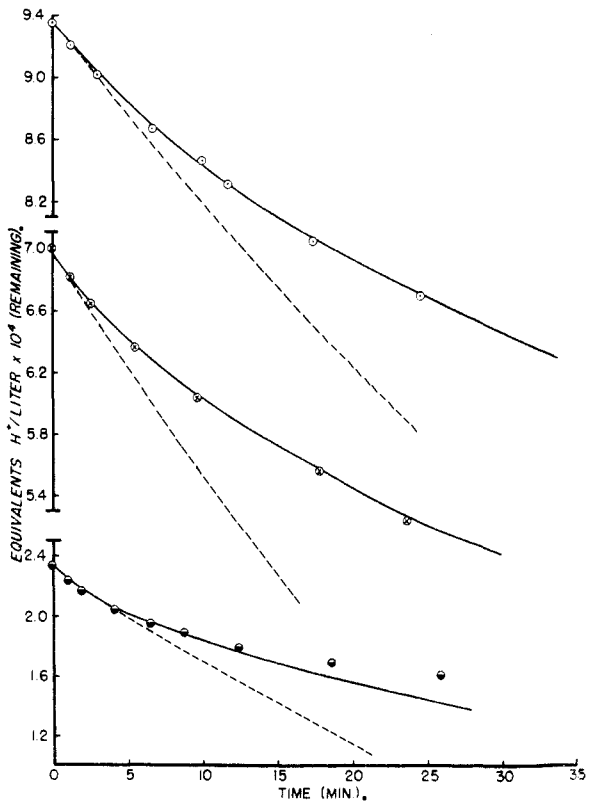

Fig. 2.—All circles represent experimental values. The solid lines are theoretical curves derived from a competitive inhibition mechanism. The broken lines are calculated for an uninhibited reaction.

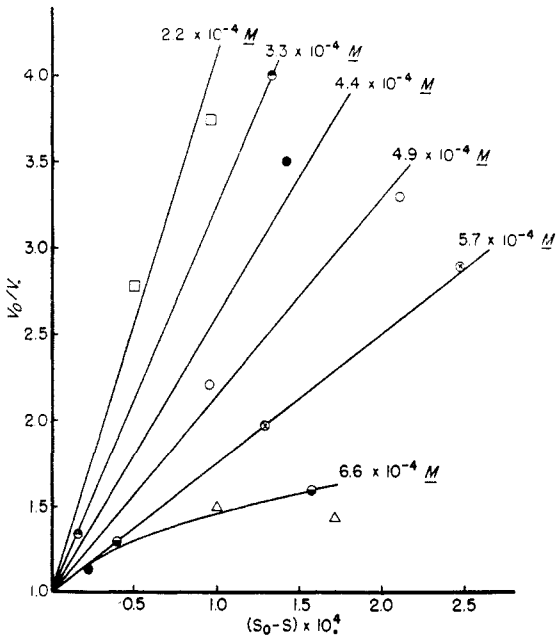

Fig. 3.—v_0 represents the rate calculated on the basis that the products do not inhibit the reaction (equation 1); v is the observed (inhibited) rate. The inhibitor concentration was taken as proportional to $S_0 - S$.

Discussion

The observed maximum shown in Fig. 1 indicates that excess substrate inhibits the reaction. The typical Michaelis-Menten curve would reach a limiting value ($V_{max} = k(E)$) asymptotically. Kunitz[2] observed a similar maximum in measuring the extent of acid precipitability with time and tentatively suggested that the viscosity of the solution might be involved. Viscosity cannot be the cause of the observed maximum (as is also intimated by Kunitz) since, in our case at least, a relative viscosity of about 2 or 3 is reached in less than a minute. To explore this effect further, dye-binding experiments were carried out with DNA. The results clearly show that the number of available sites does not decrease with increasing DNA concentration in the region of the maximum observed in the rate studies. Thus it is highly probable that no association occurs among the phosphate anions of DNA capable of binding dye in such a way that the sites are unavailable.[5]

However, no definite conclusion can be drawn concerning association among sites which are susceptible to enzyme action. The situation is also complicated by the fact that the apparent size and shape of DNA (*e.g.*, coiling or uncoiling) varies with concentration and susceptibility to DNAase action may also be altered.[6-9] The kinetic derivations which follow apply to the middle concentration range of DNA. In the very low and high re-

(5) L. F. Cavalieri and A. Angelos, THIS JOURNAL, **72**, 4686 (1950).
(6) S. Basu, *Nature*, **168**, 431 (1951).
(7) G. Jungner, I. Jungner and L. G. Allgen, *ibid.*, **163**, 849 (1949).
(8) J. Pouyet, G. Scheibling and H. Schwander, *J. Chem. Phys.*, **47**, 716 (1950).
(9) In the present investigation a plot of reduced viscosity *vs.* concentration showed a break in the region of $S_0 = 8 \times 10^{-4}$ *M*; this suggests that a change in the macromolecular structure of DNA occurs at this concentration.

gions, the initial rate, v_0, is not proportional to the enzyme concentration; this anomaly is due presumably to alterations in the macromolecular structure of DNA. Our treatment of the variation of v_0 with $[S_0]$ may therefore be regarded as a simplification.

Inhibition by substrate may be interpreted in terms of a competition between substrate and water molecules for sites on the enzyme. This is eminently reasonable since the cleavage of a sugar-phosphate bond does involve a molecule of water and it is probable that both the phosphate and water must be adsorbed on neighboring sites of the enzyme for reaction to occur. The theoretical curve shown in Fig. 1 was not constructed on the basis of any particular enzyme model. Although it would have been possible to do so, it would be a matter of great difficulty to prove that any one model, or sets of models, exclusively represented the situation.

Equations for various model reaction systems were developed to yield a maximum in the v_0 *vs.* S_0 plot. The simplest one giving a satisfactory fit is

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_3} E + P$$

$$ES_1 + (n-1)S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ES_n$$

where ES_1 is the active Michaelis–Menten type intermediate and ES_n is the inactive substrate complex.[10] In the present case the smallest value of n which gave a satisfactory fit was 3. The accompanying differential equation, after making the usual assumptions and substitutions, is

$$-\frac{d(S)}{dt} = v_0 = \frac{k_3(E)K_1(S)}{1 + K_1(S) + K_1K_2(S)^n} \qquad (1)$$

where E = total enzyme concentration; S = total substrate concentration; $K_1 = k_1/(k_2 + k_3)$; $K_2 = k_4/k_5$.

Values for the three constants k_3, K_1 and K_2 were obtained explicitly, on the basis that $n = 3$, in the following manner. The intercept of a plot of S_0/v_0 *vs.* S_0 yielded $k_3(E)K_1$. Equation (1) was rearranged to

$$K_2 = \frac{k_3(E)K_1}{K_1v_0(S)^{n-1}} - \frac{1}{K_1(S)^n} - \frac{1}{(S)^{n-1}} \qquad (2)$$

Using two sets of values for S_0 and v_0 and eliminating K_2 from equation (2) yielded K_1. Substitution of K_1 in (1) gave K_2. It turns out that K_1 is very sensitive to $k_3(E)K_1$, since K_1 is obtained as a difference between two large numbers. Since our data do not include initial rates at very low substrate concentrations, extrapolation to obtain the intercept, $k_3(E)K_1$, resulted in a somewhat uncertain value. With a value for the intercept at 0.0252, $S_1 = 3.51 \times 10^{-4}$, $v_1 = 8.0 \times 10^{-6}$ and $S_2 = 4.68 \times 10^{-4}$, $v_2 = 9.5 \times 10^{-6}$; k_3, K_1 and K_2 were calculated to be, respectively, 2.00×10^4, 41 and 5.2×10^7. The theoretical curve shown in Fig. 1 was obtained using $k_3 = 2.22 \times 10^4$, $K_1 = 41$ and $K_2 = 3.7 \times 10^7$. Implicit in this treatment is the assumption that all sites pertaining to K_1 (or K_2) are identical and the velocity constant, k_3, for the decomposition of ES_1 is the same for all such complexes. It is clear, however, that neither the sites of the substrate nor those of the enzyme need be such that only one set of constants represents the actual situation. It would be more generally true that k_3, K_1 and K_2 are each representative of a group of similar but not necessarily identical sites.

It may readily be shown that the order of the reaction with respect to time is greater than that with respect to concentration. This behavior is characteristic of systems which are inhibited by the products of reaction.[11] This conclusion was confirmed qualitatively by preparing the products independently from a DNA solution which had been treated with enzyme for an extended period. After concentrating this reaction mixture, aliquots were added to the reaction system and a definite decrease in the initial rate was noted. This experiment, slightly modified, was repeated quantitatively and found to agree with the calculated results.

It is apparent immediately that the multiplicity of products renders the interpretation of their effects on initial rates a difficult problem. In the experiments under discussion, the concentration of inhibitor, I, present at any time, t, was assumed to be proportional to $(S_0 - S)$; where S is the substrate concentration at t. In order to be able to plot v_0/v against I, runs were carried out at successive S_0 concentrations such that there was overlapping in S values. That is, this procedure enables the calculation of rates at two or more inhibitor concentrations for one substrate concentration. The results are contained in Fig. 3. In these calculations it is assumed that the products at both substrate concentrations are identical.

To substantiate the procedure used in obtaining the v_0/v *vs.* I plot and to support the assumption that the inhibition products are essentially identical during the initial phases of the various runs, a number of experiments were carried out. A reaction was allowed to proceed to a definite value of acid production. This mixture was then supplemented with fresh reaction mixture such that the original initial concentration of DNA was restored. The resultant mixture therefore differed only in that inhibitor products were present. The ratio v_0/v thus obtained was in agreement with the comparable ratio calculated in the manner described above. In Fig. 3, for example, the squares at 2.2×10^{-4} M and the triangles at 6.6×10^{-4} M were determined by the modified experimental procedure just described.

The equation corresponding to (1) for competitive and non-competitive inhibition are, respectively

$$v = \frac{k_3(E)K_1(S)}{1 + K_1(S) + K_1K_2(S)^3 + K_3(I)} \qquad (3)$$

$$v = \frac{k_3(E)K_1(S)}{1 + K_1(S) + K_1K_2(S)^3 + K_3(I) + K_1K_3(S)(I)} \qquad (4)$$

where K_3 is the inhibitor equilibrium constant. Dividing (1) by (3) yields (5); dividing by (4) yields (6).

(10) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 659 (1934).

(11) M. Letort, Thesis, Paris, 1937; *cf.* K. J. Laidler and J. P. Hoare, THIS JOURNAL, **71**, 2699 (1949).

$$v_0/v = 1 + \frac{K_3(I)}{1 + K_1(S) + K_1K_2(S)^3} \quad (5)$$

$$v_0/v = 1 + \frac{K_3(I)(K_1(S) + 1)}{1 + K_1(S) + K_1K_2(S)^3} \quad (6)$$

A plot of v_0/v *vs.* I at constant S gives a slope of $K_3/(1 + K_1(S) + K_1K_2(S)^3)$ for competitive inhibition (equation 5), and $(K_1(S) + 1)K_3/(1 + K_1(S) + K_1K_2(S)^3)$ for non-competitive inhibition (equation 6). K_3 may be calculated from each slope but (5) cannot be distinguished from (6). In each case the slope decreases with increasing substrate concentration. At high S values $K_3 = 1 \times 10^{+4}$; at low, $K_3 = 3 \times 10^{+4}$.

By use of the integrated forms of equations (3) and (4), S *vs.* t curves were calculated using the values given above for k_3, K_1 and K_2 and a value of 1.3×10^4 for K_3. The unbroken lines in Fig. 2 are the resulting theoretical curves for the inhibited reaction using the integrated form of (3). The various circles are experimentally determined points, while the broken line is the curve which would result if the products of reaction caused no inhibition; these were calculated using the integrated form of equation (1). The agreement is seen to be good except at lower substrate concentrations where there is evidence for more inhibition than can be accounted for on the basis of a simple competitive mechanism. Better agreement was realized if K_3 and k_3 were treated as parameters but since this procedure has questionable theoretical significance the best fits are not included. It might be postulated that the inhibition products are different at low substrate concentrations, but apparent deviations in inhibitor constants have been encountered[12] where the products of reaction are simple and would not be expected to account for the discrepancy.

Theoretical curves obtained by the use of the integrated form of (4) resulted in poor agreement with the experimental points. On this basis it is tentatively suggested that the inhibition is of a competitive nature.

In considering the kinetics of the inhibition by substrate and by the products of reaction, it appears likely that the doubly charged phosphate anion is responsible to some extent for both types of inhibition. The doubly charged phosphate anion is a product of enzymatic hydrolysis and it is probable that this group is involved in the inhibition. Since the original DNA contains this group to the extent of about 5%[4,13] the possibility exists that in this case the inhibition may also be caused by the doubly charged anion. A calculation in support of this hypothesis involves a comparison of the ratio uninhibited rate/inhibited rate for the substrate inhibition with that for the inhibition by products. Ratios for the latter are contained in Fig. 3. For the former, the uninhibited rate curve corresponding to Fig. 1 was approximated using the usual relation

$$v = k_3(E)K_1(S)/(1 + K_1(S)) \quad (7)$$

With the values for k_3 and K_1 given above, the value of v_0/v at 5.7×10^{-4} M is calculated to be 1.28. This corresponds to 0.91×10^{-4} M of double anions in the original DNA. From Fig. 3 the value of v_0/v at 5.7×10^{-4} M and 0.91×10^{-4} M for $(S_0 - S)$ is 1.69. This is fair agreement considering the approximate method of calculation. If not all of the end-groups were available in the original DNA (perhaps because of hydrogen bonding), the agreement would be better. For example, if only one-half of the end groups were available for inhibition the comparable v_0/v ratio for inhibition by products obtained from Fig. 3 would be 1.32 (on the basis of $(S_0 - S) = 0.45$), which is in good agreement with 1.28 for the substrate inhibition ratio.

In further support of the suggestion that the doubly charged phosphate anion is an inhibitor is the fact that the ratio K_3/K_1 is about 300. It will be recalled from dye-binding experiments[5,14] that the ratio k_1/k_2 (intrinsic binding constant for doubly charged phosphate anion/intrinsic binding constant for singly charged phosphate anion) was of this order of magnitude. That is, the association constant K_1 (which is not a true equilibrium constant but may be taken as a measure of affinity[15]

$$K_1 = (ES)/(E_{free})(S)$$

would correspond to the interaction between singly charged phosphate anion and DNAase, while K_3 would represent the interaction of the doubly charged groups.

It must be noted that this type of substrate inhibition is competitive, *i.e.*, that the doubly charged groups of DNA compete for sites on the enzyme which are normally occupied by singly charged groups. The competition by substrate for sites normally occupied by water (noted earlier) is non-competitive in nature. The competitive inhibition is represented by the equation

$$v_0 = \frac{k_3(E)K_1(S)}{1 + K_1(S) + K_2'(S')^3}$$

This equation is based on the sequence

$$\begin{aligned} &E + S \rightleftarrows ES \longrightarrow E + P \\ &E + 3S' \rightleftarrows ES_3' \text{ (inactive)} \end{aligned} \quad (8)$$

where K_1 has the significance stated above, K_2' is the substrate inhibition constant involving the doubly charged anions and S' is the substrate concentration in terms of the doubly charged anions (*i.e.*, *ca.* 5% of the total DNA phosphate). A theoretical curve almost identical to that shown in Fig. 1 is obtained if a value of 2.0×10^{11} is used for K_2. If it is assumed that the three association constants implied in equation 8 are equal, the value of each is $(K_2')^{1/3} = 0.58 \times 10^4$ M. This compares favorably with K_3, the inhibition constant for the products.

We suggest, therefore, that both competitive and non-competitive inhibition by substrate could occur; these interactions would take place at different sites on the enzyme. The relative extent of each type is a matter of conjecture at present.

The analysis just developed suggests that the type of phosphate anion is of importance both in enzymatic activity (singly charged groups) and in inhibition (doubly charged groups). However, in

(12) K. M. Harmon and C. Niemann, *J. Biol. Chem.*, **178**, 743 (1949).

(13) L. F. Cavalieri, THIS JOURNAL, **74**, 1242 (1952).

(14) L. F. Cavalieri, A. Angelos and M. E. Balis, *ibid.*, **73**, 4902 (1951).

(15) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

addition to electrostatic effects, structural characteristics doubtlessly are of significance. This phase of the problem is now under investigation.

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Hydantoins as Anticonvulsants. I. 5-R-5-(2-Thienyl)-hydantoins[1]

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The synthesis of nineteen 5-substituted-5-(2-thienyl)-hydantoins and nine 3-alkyl- or 1,3-dialkyl-5-substituted-5-(2-thienyl)-hydantoins is described in this work. The compounds were tested elsewhere for anticonvulsant activity and the results are reported. A few of the compounds were of the same order of activity as 5,5-diphenylhydantoin (dillantin). N-Alkylation reduced anticonvulsant activity in every case.

There has been in progress in this Laboratory during the past several years a program which has for its purpose the study of the effect of varied substitution of the hydantoin nucleus on its anticonvulsant activity. This paper is the first of a series which describes the synthesis and gives the results of testing elsewhere of these compounds.

At the St. Louis Meeting of the American Chemical Society in 1941, there were described five examples of 5-substituted-5-(2-thienyl)-hydantoins. These and certain others were patented in 1945.[2] In 1945, Chabrier and Tchoubar[3] reported the synthesis of 5-ethyl-5-(2,5-dimethyl-3-thienyl)-hydantoin, 5-methyl-5-(2,5-dimethyl-3-thienyl)-hydantoin and 5-methyl-5-(5-methyl-2-thienyl)-hydantoin. The latter compound was reported in low yield (5–10%), and these authors report that under the conditions used they were unable to synthesize 5-methyl-5-(2-thienyl)-hydantoin. In 1949, Long and Miller reported the synthesis of a series of 1-alkyl- or/and -aryl-5-(2-thienyl)-hydantoins.[4] In 1949, also, Bywater and Coleman[5] were issued a patent relating to 5,5-di-(2-thienyl)-hydantoin.

The compounds described in the present work were prepared by a modification of the method of Bucherer[6] in which the appropriate ketone is heated with ammonium carbonate and potassium cyanide

TABLE I

ALKYL 2-THIENYL KETONES, R(C_4H_3S)CO

R	B.p., °C. at atm. press.	d^{20}_4	$n^{20}D$	Yield, %
Methyl[a]	214	1.1711	1.5652	63
Ethyl[b]	227	1.1305	1.5533	70
n-Propyl[c]	240	1.0941	1.5434	74
i-Propyl[d]	228	1.0894	1.5405	72
n-Butyl[e]	258	1.0664	1.5357	76
i-Butyl[f]	245	1.0619	1.5330	73
n-Amyl[g,h]	275	1.0473	1.5301	80
i-Amyl[i]	267	1.0419	1.5273	83

[a] A. Peter, *Ber.*, **17**, 2643 (1884). [b] K. Krekeler, *ibid.*, **19**, 677 (1886). [c] W. Steinkopf and I. Shubart, *Ann.*, **424**, 10 (1920); H. Scheibler and F. Rettig, *Ber.*, **59**, 1194 (1926), report d^{20}_{20} 1.0730, $n^{20}D$ 1.52418. [d] Krekeler, ref. *b*, p. 675. [e] P. Cagniant and A. Deluzarche, *Compt. rend.*, **223**, 1149 (1946). [f] Steinkopf and Shubart, ref. *c*, p. 11. [g] Cagniant and Deluzarche, *Compt. rend.*, **225**, 456 (1947), report d^{17}_4 1.0463, $n^{17}D$ 1.5299; E. Campaigne and J. L. Diedrich, THIS JOURNAL, **70**, 392 (1948), report d^{20}_4 1.065, $n^{20}D$ 1.5301. [h] Fifty-five per cent. of the anticonvulsant activity of dillantin. Electroshock test in cats; equal doses of 50 mg./kg. [i] Calcd. for $C_{10}H_{14}OS$: S, 17.59. Found: S, 17.45.

TABLE II

ARYL, ARALKYL AND CYCLOALKYL 2-THIENYL KETONES, RR'CO

R	R'	M.p., °C.	Yield, %	Anticonvulsant activity[m]
Phenyl	2-Thienyl[a]	55.5–56	86	30
Cyclohexyl	2-Thienyl[b]	43–43.5	88	
4-Methylphenyl	2-Thienyl[c]	75–76	93	0
Benzyl	2-Thienyl[d]	49–50	86	20
2-Phenylethyl	2-Thienyl[e]	45–45.5	88	
Benzhydryl	2-Thienyl[f]	134.5–136	55	30
Phenyl	5-Methyl-2-thienyl[g,h]	Oil	54	
Phenyl	4-Methyl-2-thienyl[i]	91–92	81	
4-Fluorophenyl	2-Thienyl[j]	95.5–96	45	
Phenyl	5-Chloro-2-thienyl[k]	48.5–49.5	76	
Phenyl	5-Bromo-2-thienyl[l]	75.5–76	76	

[a] A. Comey, *Ber.* **17**, 790 (1884). [b] B.p. 142–144° (4 mm.). Calcd. for $C_{11}H_{14}OS$: S, 16.50. Found: S, 16.48. [c] W. Steinkopf and M. Bauermeister, *Ann.*, **403**, 71 (1914). [d] P. Cagniant and A. Deluzarche, *Compt. rend.*, **223**, 1150 (1946), report m.p. 44.5°. [e] B.p. 189–191° (9 mm.). Calcd. for $C_{13}H_{12}OS$: S, 14.80. Found: S, 14.69. [f] Calcd. for $C_{18}H_{14}OS$: C, 77.67; H, 5.07. Found: C, 77.74; H, 5.22. [g] B.p. 142–144° (2.5 mm.). [h] J. Volhard, *Ann.*, **267**, 182 (1892), reports m.p. 124°. Ernst, *Ber.*, **19**, 3280 (1886), reports the substance is an oil. [i] W. Steinkopf and H. Jacob, *Ann.*, **515**, 281 (1935). [j] B.p. 142–145° (4 mm.). Calcd. for $C_{11}H_7OSF$: C, 64.06; H, 3.42. Found: C, 64.41; H, 3.76. [k] B.p. 153–155° (4 mm.). Calcd. for $C_{11}H_7OSCl$: S, 14.37. Found: S, 14.41. [l] A. W. Weitkamp and C. S. Hamilton, THIS JOURNAL, **59**, 2701 (1937). [m] Per cent. of activity of dillantin. Equal doses of 50 mg./kg. in cats by electroshock method.

(1) Presented at the 117th Meeting of the American Chemical Society at Philadelphia, Pa., 1950.

(2) James J. Spurlock, U. S. Patent 2,366,221, Jan. 2, 1945

(3) P. Chabrier and B. Tchoubar, *Compt. rend.*, **220**, 284 (1945); see also P. Chabrier, B. Tchoubar and S. LeTellier-Dupre, *Bull. soc. chim.*, 332 (1946).

(4) L. M. Long and C. A. Miller, THIS JOURNAL, **71**, 669 (1949).

(5) W. G. Bywater and W. R. Coleman, U. S. Patent 2,468,168, April 26, 1949.

(6) H. T. Bucherer and V. A. Lieb, *J. prakt. Chem.*, [2] **141**, 5 (1934).