occurs. Use may be made of some data obtained by Niemann[32] to answer this question. The Michaelis–Menten constants for L-tryptophanamide and acetyl L-tryptophanamide are 6.3×10^{-3} M and 5.3×10^{-3} M, and the inhibitor constants for the D-enantiomorphs of these compounds are 3.2×10^{-3} and 2.7×10^{-3} M, respectively. In these systems there is evidence that $k_2 >> k_3$, so that K_m may be used as a measure of enzyme–substrate affinity. If this is so, introduction of an acyl group gives a change of only 10% in the formation of the enzyme–substrate complex or the enzyme–inhibitor complex. On the other hand the k_3 values for L-tryptophanamide and acetyl-L-tryptophanamide are 0.006×10^{-3} and 0.50×10^{-3} mole/l./min./mg. enzyme N/ml., a ratio of eighty-three. It is apparent that the large difference in reactivity of the two compounds is in the second step. Introduction of the acyl group would be expected to polarize the nitrogen–hydrogen bond and make the hydrogen atom more able to enter into hydrogen bonding. If an extrapolation can be made from the tryptophan amide system to the benzoyl-L-phenylalanine ethyl ester system ($k_2 >> k_3$), the high first-order velocity constant for this ester over its oxygen homomorph suggests that the hydrogen bonding effect is to be found in the breakdown of the intermediate complex and not in its formation.

(32) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

If hydrogen bonding plays an important role in the breakdown of the enzyme–substrate into products, the following may be suggested: (1) hydrogen bonding to the enzyme "surface" is not important since the complex has already formed by the time hydrogen bonding becomes important; (2) hydrogen bonding to a molecule of water may be important; (3) the water molecule appears in the enzymatic hydrolysis not as a part of the enzyme–substrate complex but as a reactant in the subsequent decomposition of the complex into products. The last conclusion is not only a consequence of the above data but also of the concept of competitive inhibition and is confirmed by the lack of oxygen exchange with carboxylic acids which inhibit enzymatic hydrolysis.[33] The specification of the role of the water molecule in enzymatic hydrolysis seems to have been largely ignored except by several workers who have included the water molecule in the enzyme–substrate complex.[34,35]

Acknowledgment.—The authors wish to express their gratitude to Armour and Co. for a generous gift of α-chymotrypsin.

(33) M. L. Bender and K. C. Kemp, unpublished results.

(34) M. L. Barnard and K. J. Laidler, THIS JOURNAL, **74**, 6099 (1952); K. J. Laidler and J. P. Hoare, *ibid.*, **71**, 2699 (1949).

(35) C. G. Swain and J. F. Brown, Jr., *ibid.*, **74**, 2540 (1952).

CHICAGO, ILLINOIS

[CONTRIBUTION NO. 1982 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Effect of Added Sodium or Potassium Chloride upon the α-Chymotrypsin Catalyzed Hydrolysis of Chloroacetyl-L-tyrosinamide in Aqueous Solutions at 25° and pH 7.75[1]

BY HENRY J. SHINE AND CARL NIEMANN[2]

RECEIVED APRIL 2, 1955

The initial rate of the α-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.75 and 0.02 M in the THAM component of a THAM-HCl buffer is increased by the addition of either sodium or potassium chloride. The increase in initial velocities is given by the relation $\log (v_0'/v_0^0) = 0.30 \sqrt{M}$ where M is the molarity of the reaction mixture with respect to added sodium or potassium chloride. From a study of the effect of added sodium chloride in systems in which the concentration of the specific substrate was varied it was found that the value of K_S is essentially independent of the concentration of added sodium chloride whereas the value of k_3 is increased by the amount given by the relation $\log (k_3/k_3^0) = 0.30 \sqrt{M}$.

In 1950 Jandorf[3] noted that the addition of magnesium sulfate to systems containing α-chymotrypsin and acetyl-L-tyrosine ethyl ester led to a proportionality between the enzyme concentration and the so-called esterase activity which was not observed in the absence of added magnesium sulfate and that the addition of this salt in a sufficient but otherwise unspecified amount caused a fivefold increase in the apparent esterase activity of the enzyme over that observed in a control experiment where presumably a lesser amount of magnesium sulfate was present. However, this investigator also reported that "no salt which was found to potentiate the esterase activity of α-chymotrypsin had an effect on the proteinase (casein hydrolysis) activity of the enzyme" thus leaving the impression, intended or not, that potentiation by added salt is to be observed only with respect to apparent esterase activity. Two years later Shine and Niemann[4] noted that the rate of the α-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-tyrosinamide, in aqueous solutions at 25° and pH 7.75, increased with increasing concentration of added magnesium sulfate thus demonstrating the generality of an apparent positive salt effect in systems involving α-chymotrypsin and either an amide or an ester type of specific substrate. In the same year Neurath and his coworkers[5] reported that the addition of calcium ion to systems containing α-chymotrypsin and either an ester or an amide type of specific substrate caused an increase in the activity of the enzyme and it was further stated that calcium ion was far more

(1) Supported in part by a grant from Eli Lilly and Co.

(2) To whom inquiries regarding this article should be sent.

(3) B. J. Jandorf, *Federation Proc.*, **9**, 186 (1950).

(4) H. J. Shine and C. Niemann, THIS JOURNAL, **74**, 97 (1952).

(5) M. M. Green, J. A. Gladner, L. W. Cunningham, Jr., and H. Neurath, *ibid.*, **74**, 2122 (1952).

effective in producing this result than was magnesium or a number of other divalent cations thus implying that with calcium ion a specific ion effect was involved.

Although all of the experiments discussed above were of a preliminary nature it was clear that one could anticipate a general positive salt effect and a possible specific ion effect upon the addition of certain salts to systems containing α-chymotrypsin and characteristic specific substrates of this enzyme. As it appeared that a specific ion effect might be masked, at least in part, by a general positive salt effect, it was decided to first investigate the effect produced by the addition of sodium or potassium chloride to a typical α-chymotrypsin-catalyzed reaction since such an investigation would also be of value in anticipating the consequence of variation of the concentration of the frequently employed uni-univalent THAM[6]–HCl buffer[7] and of the possible error introduced in inhibition studies where univalent charged competitive inhibitors had been added to the reaction systems, in the form of their uni-univalent salts.[4,7–9]

The results obtained from experiments which involved the addition of increasing amounts of sodium chloride to a reaction system containing α-chymotrypsin and chloroacetyl-L-tyrosinamide in aqueous solution at 25° and *p*H 7.75 and buffered with a THAM–HCl buffer, which was 0.02 M in the THAM component and 0.01 M in hydrochloric acid, under conditions where the initial specific substrate concentration was maintained at 20×10^{-3} M and the enzyme concentration at 0.0677 mg. protein-nitrogen/ml. are summarized in Table I. It should be noted that the initial velocities given in this table are the corrected initial velocities which were obtained from plots of $([S]_0 - [S]_t)$ *vs.* t and of $\ln [S]_0/[S]_t$ *vs.* t as described by Jennings and Niemann.[10]

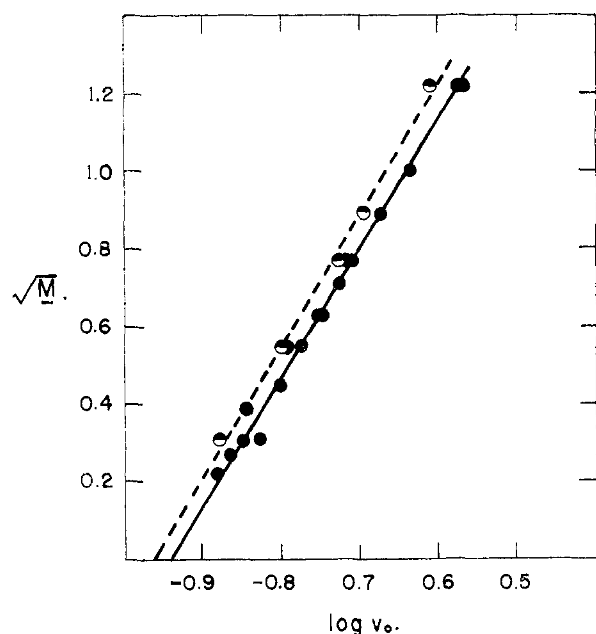


Fig. 1.—Effect of added sodium or potassium chloride on the α-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.75 and 0.02 M in the THAM component and 0.01 M in the HCl component of a THAM–HCl buffer; $[S]_0 = 20 \times 10^{-3}$ M; $[E]$ = 0.0677 mg. protein-nitrogen/ml.; ●, sodium chloride; ◓, potassium chloride; solid line $\log v_0 = (-0.944 + 0.304)\sqrt{M}$; broken line $\log v_0 = (-0.961 + 0.298)\sqrt{M}$; v_0 in units of 10^{-3} M/min.

TABLE I

EFFECT OF ADDED SODIUM OR POTASSIUM CHLORIDE ON THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF CHLOROACETYL-L-TYROSINAMIDE[a]

		v_0 [c,d]		$-\log v_0$	
M [b]	$\sqrt{M}$	NaCl	KCl	NaCl	KCl
0.05	0.224	0.131	...	0.883	...
.075	.274	.136	...	.867	...
.10	.316	.143	0.133	.845	0.876
.10	.316	.150	.132	.824	.879
.10	.316	...	.133	...	.876
.15	.387	.143	...	.845	...
.20	.447	.158	...	.801	...
.30	.548	.169	.166	.772	.780
.30	.548	.169	.160	.772	.796
.30	.548	.161	.162	.793	.791
.30	.548	.168	...	.775	...
.40	.633	.181	...	.742	...
.40	.633	.176	...	.755	...
.40	.633	.175	...	.757	...
.50	.707	.187	...	.728	...
.60	.775	.196	.189	.708	.724
.60	.775	.196	.190	.708	.721
.60	.775	.192	...	.717	...
.80	.894	.212	.203	.674	.693
1.0	1.0	.233	...	.633	...
1.50	1.225	.264	.243	.578	.614
1.50	1.225	.269	...	.570	...

[a] In aqueous solutions at 25° and *p*H 7.75 and 0.02 M in the THAM component and 0.01 M in the HCl component of a THAM–HCl buffer with $[S]_0 = 20 \times 10^{-3}$ M and an enzyme concentration of 0.0677 mg. protein-nitrogen/ml. [b] Molarity of reaction system with respect to added sodium or potassium chloride. [c] In units of 10^{-3} M/min. [d] Corrected values evaluated from plots of $([S]_0 - [S]_t)$ *vs.* t and $\ln [S]_0/[S]_t$ *vs.* t by the method of Jennings and Niemann.[10]

It will be seen from the plot given in Fig. 1 that when the square root of the molarity of the added sodium chloride is plotted against the logarithm of the initial velocity one obtains a straight line within the limits of experimental error. A least squares fit of the data to the relationship $\log v_0 = a + b\sqrt{M}$ resulted in a value of $a = -0.9442$, with a probable error of ±0.0032, and a value of $b = 0.3040$, with a probable error of ±0.0049. Since the value of v_0 when $M = 0$ is the antilogarithm of −0.9442, *i.e.*, 0.114×10^{-3} M/min., it is of interest to compare this value with that calculated from a value of $K_S = 27 \pm 2 \times 10^{-3}$ M,[7] a value of $k_3 = 4.0 \pm 0.2 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml.,[7] a value of $[S]_0 = 20 \times 10^{-3}$ M and a value of $[E]$ = 0.0677 mg. protein-nitrogen/ml., *i.e.*, $0.115 \pm 0.010 \times 10^{-3}$ M/min. The good agreement of these two values of v_0 allows one to conclude that the results of the present study are consistent with those obtained earlier.[4,7] Finally, from the value of

(6) Tris-(hydroxymethyl)-aminoethane.

(7) R. J. Foster and C. Niemann, THIS JOURNAL, **77**, 1886 (1955).

(8) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).

(9) R. J. Foster and C. Niemann, *ibid.*, **77**, 3370 (1955).

(10) R. J. Jennings and C. Niemann, *ibid.*, **75**, 4687 (1953).

b given above it is possible to express the effect of added sodium chloride, under the specified conditions, in terms of the relatively simple relationship $\log (v_0/v_0^0) = (0.304 \pm 0.005)\sqrt{M}$ in which v_0^0 is the initial velocity in the absence of added sodium chloride.

A second series of experiments comparable to those described immediately above was conducted with potassium chloride and the results of these experiments, which were evaluated in the same manner as those of the first series, are also summarized in Table I. As with the experiments involving sodium chloride a plot of $\log v_0$ *vs.* $\sqrt{M}$ led to a straight line within the limits of experimental error, *cf.* Fig. 1, and a least squares fit of the data to the relation $\log v_0 = a + b\sqrt{M}$ resulted in a value of $a = -0.9614$, with a probable error of ± 0.0062, and a value of $b = 0.2977$, with a probable error of $\pm$ 0.0091. A value of $\log v_0$ of $-$ 0.9614 for M = 0 corresponds to a value of $v_0 = \pm 0.109 \times 10^{-3}$ M/min. While this value is lower than the value of v_0 for $M = 0$ observed in the sodium chloride experiments, *i.e.*, 0.114 $\times$ 10^{-3} M/min., the difference between these two values is not significant and both are well within the limits of the value of $v_0 = 0.115 \pm 0.010 \times 10^{-3}$ M/min. calculated above. Thus, we may conclude that the experiments with both sodium and potassium chloride resulted in values of v_0^0 which were identical within the limits of probable error and that both of these values were identical within the limits of experimental error, with the value of v_0^0 expected on the basis of previously determined or known parameters of the system under investigation.

The value of b derived from the experiments with potassium chloride, *i.e.*, 0.2977 $\pm$ 0.0091, was identical, within the limits of probable error, with that observed for the case of added sodium chloride, *i.e.*, 0.3040 $\pm$ 0.0049, and we may summarize the effect produced by added sodium or potassium chloride in the reaction system specified above by the relation given in equation 1

$$\log (v_0/v_0^0) = (0.30 \pm 0.01)\sqrt{M} \qquad (1)$$

when M, the molarity of the reaction system with respect to added sodium or potassium chloride, is between the limits of 0 and 1.5 M. The magnitude of the effect to be observed can be appreciated by the fact that equation 1 predicts that the presence of 0.01 M sodium or potassium chloride will cause an increase in v_0^0 of 7%, 0.1 M an increase of 24%, 0.2 M an increase of 36%, 0.3 M an increase of 46%, 0.5 M an increase of 63% and 1.0 M an increase of 100%. While it is not known whether equation 1 can be used to extrapolate beyond concentrations of 1.5 M it may be noted that such an extrapolation would lead to an expected increase of v_0^0 of the order of four- to fivefold for reaction systems which are saturated, or nearly so, with sodium or potassium chloride.

Having established the validity of equation 1 under conditions of constant specific substrate concentration attention was next directed to a study of the effect of added sodium chloride upon systems of α-chymotrypsin and chloroacetyl-L-tyrosinamide in which the specific substrate concentration was varied. In the first set of such experiments the concentration of added sodium chloride was maintained at 0.3 M and the specific substrate concentration was varied from 10 to 30 $\times$ 10^{-3} M with all other conditions being identical with those used previously. As before the primary data were evaluated by the method of Jennings and Niemann[10] and the corrected initial velocities so obtained are given in Table II. When these found values of v_0 are compared with the values of v_0 which were calculated by means of equation (1) from the corresponding values of v_0^0, which in turn were calculated from the previously determined values of K_S and k_3[4,7] and the other known parameters of the system under investigation it will be seen that the two sets of values of v_0 are in good agreement. Thus, we may conclude that the relation given by equation 1 is independent of the concentration of the specific substrate, at least for the particular uncharged specific substrate that was used in these studies and over the range of concentrations that were employed.

TABLE II

EVALUATION OF K_S AND k_3 FOR CHLOROACETYL-L-TYROSINAMIDE IN THE PRESENCE OF 0.3 M NaCl[a]

$[S]_0$[b]	v_0^0, calcd.[c]	v_0 Calcd.[d]	v_0 Found[e]	$[S]_0/v_0$ Calcd.	$[S]_0/v_0$ Found
10	0.073	0.107	0.106	93.5	94.3
12.5	.086	.126	.131	99.0	95.2
12.5	.086	.126	.121	99.0	103.1
15	.097	.142	.153	105.6	98.0
15	.097	.142	.141	105.6	106.4
20	.115	.168	.169	119.0	118.3
20	.115	.168	.169	119.0	118.3
20	.115	.168	.161	119.0	124.2
20	.115	.168	.168	119.0	119.0
25	.130	.190	.185	131.6	135.1
30	.142	.207	.204	144.9	147.1

[a] In aqueous solutions at 25° and pH 7.75 and 0.02 M in the THAM component and 0.01 M in the HCl component of a THAM–HCl buffer with an enzyme concentration of 0.0677 mg. protein-nitrogen/ml. [b] In units of 10^{-3} M. [c] Calcd. from a value of $K_S = 27 \pm 2 \times 10^{-3}$ M and a value of $k_3 = 4.0 \pm 0.2 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. [d] Calcd. from the relation $\log (v_0/v_0^0) = 0.30\sqrt{0.3}$. [e] Corrected values evaluated from plots of $([S]_0 - [S]_t)$ *vs.* t and $\ln [S]_0/[S]_t$ *vs.* t by the method of Jennings and Niemann.[10]

The data that are given in Table II may be used to evaluate K_S and k_3 for the system α-chymotrypsin–chloroacetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.75 which are 0.02 M in the THAM component and 0.01 M in the hydrochloric acid component of a THAM–HCl buffer and 0.3 M in sodium chloride. A graphical solution of these data, based upon a plot of $[S]_0/v_0$ (calculated) *vs.* $[S]_0$[11] gave, with an estimated error in the value of K_S of $\pm 7.5\%$ and an estimated error in the value of k_3 of $\pm 5\%$, the following values: $K_S = 26.5 \pm 2 \times 10^{-3}$ M, $k_3 = 5.7 \pm 0.3 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. A least squares fit of the quantities $[S]_0/v_0$ (found) and $[S]_0$ to the line $y = a + bx$ led to a value of $a = 63.4$, with a probable error of ± 2.6, and a value of $b = 2.81 \times 10^3$, with a probable error of $\pm 0.13 \times 10^3$. With $a = K_S/k_3$ [E] and $b = 1/k_3$ [E] it follows that in this instance

(11) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

$K_S = 23 \pm 3 \times 10^{-3}$ M and $k_3 = 5.3 \pm 0.3 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml.

When it is recalled[7] that for the system α-chymotrypsin–chloroacetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.75 and 0.02 M in the THAM component and 0.01 M in the hydrochloric acid component of a THAM–HCl buffer $K_S = 27 \pm 2 \times 10^{-3}$ M and $k_3 = 4.0 \pm 0.2 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. it follows that the addition of sufficient sodium chloride to the above reaction mixture to bring it to 0.3 M in sodium chloride does not cause a significant change in the value of K_S but does increase the value of k_3 by approximately 40%.

In a second set of experiments, which were, for all practical purposes, identical with those of the first set except that in the second the concentration of added sodium chloride was maintained at 0.4 M instead of at 0.3 M, it again was found that the values of v_0^0 which were observed were in satisfactory agreement with those which were calculated from values of v_0^0 with the aid of equation 1, *cf.*, Table III. A graphical solution[11] of $[S]_0/v_0$ (calculated) *vs.* $[S]_0$ based upon the values given in Table III gave, with an estimated error in K_S of $\pm 7.5\%$ and an estimated error in k_3 of $\pm 5\%$, the following values: $K_S = 26 \pm 2 \times 10^{-3}$ M, $k_3 = 6.0 \pm 0.3 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. A least squares fit of the quantities $[S]_0/v_0$ (found) and $[S]_0$ to the line $y = a + bx$ led to a value of $a = 62.0$, with a probable error of ± 6.2, and a value of $b = 2.42 \times 10^3$, with a probable error of $\pm 0.30 \times 10^3$, which in turn led to a value of $K_S = 26 \pm 5 \times 10^{-3}$ M and a value of $k_3 = 6.1 \pm 0.8 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. Thus, as before K_S remained constant, within the limits of experimental error, and k_3 was observed to increase, in this instance, by approximately 50%.

TABLE III

EVALUATION OF K_S AND k_3 FOR CHLOROACETYL-L-TYROSINAMIDE IN THE PRESENCE OF 0.4 M NaCl[a]

$[S]_0$[b]	v_0^0, calcd.[c]	v_0 Calcd.[d]	v_0 Found[e]	$[S]_0/v_0$ Calcd.	$[S]_0/v_0$ Found
12.5	0.086	0.133	0.128	94.0	98.0
15	.097	.150	.165	99.9	90.9
20	.115	.178	.191	112.4	104.7
20	.115	.178	.176	112.4	113.6
20	.115	.178	.175	112.4	114.3
30	.142	.220	.223	136.5	135.1

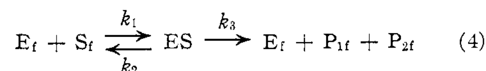
[a] In aqueous solutions at 25° and pH 7.75 and 0.02 M in the THAM component and 0.01 M in the HCl component of a THAM–HCl buffer with an enzyme concentration of 0.0677 mg. protein-nitrogen/ml. [b] In units of 10^{-3} M. [c] Calcd. from a value of $K_S = 27 \pm 2 \times 10^{-3}$ M and a value of $k_3 = 4.0 \pm 0.2 \times 10^{-3}$ M/min./mg. protein-nitrogen/ml. [d] Calcd. from the relation $\log (v_0/v_0^0) = 0.30 \sqrt{0.4}$. [e] Corrected values evaluated from plots of $([S]_0 - [S]_t)$ *vs.* t and $\ln [S]_0/[S]_t$ *vs.* t by the method of Jennings and Niemann.[10]

While we have not studied the effect of added sodium chloride under conditions wherein both the enzyme and the specific substrate concentration are varied it appears that if the results obtained in this study are capable of extrapolation and if the course of the reaction both in the absence and presence of sodium chloride can be described, in its initial stages, in terms of equation 2 one can expect that upon the addition of either sodium or potassium chloride to the system α-chymotrypsin–chloroacetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.75 and 0.02 M in the THAM component and 0.01 M in the hydrochloric acid component of a THAM–HCl buffer the value of K_S will remain unchanged and that the value of k_3 will be given, with the limits of experimental error, by equation 3 where k_3^0 is the value of the constant observed in the absence of added sodium or potassium chloride.

$$k_3[E]t = K_S \ln [S]_0/[S]_t + ([S]_0 - [S]_t) \quad (2)$$

$$\log (k_3/k_3^0) = (0.30 \pm 0.01)\sqrt{M} \quad (3)$$

The experiments reported in this communication describe a situation wherein the value of K_S remains constant, within the limits of experimental error, and the value of k_3 is increased by as much as 50%. While we are aware that, in the absence of a more complete understanding of the effect, or effects, produced by the addition of sodium chloride to the reaction system under investigation, it is impossible to arrive at a rigorous conclusion we believe that the data that have been obtained support the view that when the constants k_1, k_2 and k_3 are defined as



$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \quad (4)$$

in equation 4 that for the case at hand $k_3 << k_2$ and that $K_S = (k_2 + k_3)/k_1 \doteq k_2/k_1$.

While it appears, for the situation under discussion, that $K_S \doteq k_2/k_1$ and that K_S may be taken as an apparent dissociation constant it must be remembered[12] that even under these circumstances K_S is an index of the extent of combination of the catalytically active site of E_f with S_f in all possible modes and of the large number of species of ES presumed to be so formed there may be only a few in which the orientation of S in the ES complex is such as to lead to a transition state and the subsequent formation of E_f and free reaction products. Thus, while it may be concluded that the presence of sodium chloride appears to be without effect upon the extent of combination of E_f and S_f in all possible modes involving the participation of the catalytically active site of E_f it is entirely possible that the presence of sodium chloride may increase the probability of combination of E_f and S_f in those modes which can lead to a transition state and thus cause, in the case at hand, an increase in the value of k_3^0 without causing a significant change in the value of K_S. We do not propose to speculate, at the present time, as to how the presence of sodium chloride could produce the result described above nor do we intend to discuss other situations where the presence of sodium chloride could lead to an increase in the value of k_3^0 without causing a significant change in the value of K_S because the development of such arguments would require far more information than is now available. Because we are aware of the possibility that the effects noted in this study may be a consequence of changes in the ionic strength, or of the concentration of one

(12) R. J. Foster and C. Niemann, *Proc. Natl. Acad. Sci.*, **39**, 371 (1953).

or more of the ionic components of the reaction system we are now engaged in a study of the effects produced by other added salts not only of the type MX but also MX_2 and M_2X.

Experimental

Enzyme Experiments.—The chloroacetyl-L-tyrosinamide preparation was that which was described previously.[4] The α-chymotrypsin preparation was an Armour product, lot no. 90402. All experiments were conducted in aqueous solutions at 25° and pH 7.75. The reaction mixtures were 0.02 *M* in the THAM component and 0.01 *M* in the HCl component of a THAM–HCl buffer and of varying molarity with respect to added sodium or potassium chloride. The extent of reaction was determined titrimetrically by the method of Iselin and Niemann[13] as modified by Huang and Niemann.[14]

(13) B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **182**, 821 (1950).

(14) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

Evaluation of Experimental Data.—The data obtained from each experiment were presented in the form of $([S]_0 - [S]_t)$ *vs.* t and $\ln [S]_0/[S]_t$ *vs.* t plots and the corrected values of v_0 which were obtained from each of these plots by the method of Jennings and Niemann[10] were averaged to give the mean values of v_0 which are summarized in Tables I–III, inclusive. It was found that any given value of v_0 obtained from a corrected $([S]_0 - [S]_t)$ *vs.* t plot was in good agreement with the corresponding value of v_0 obtained from a $\ln [S]_0/[S]_t$ *vs.* t plot, in general the difference between the two values being less than ±2%. It also should be noted that all of the experiments described in this study were conducted under conditions which were compatible with the methods which were used for the evaluation of the experimental data, *i.e.*, $[E] = 1.92 \times 10^{-5}$ *M*, $E_S' = [E]/K_S = 0.07 \times 10^{-2}$ and $S_S' = [S]/K_S = 0.4$–1.2.

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[CONTRIBUTION FROM THE RESEARCH DEPARTMENT, THE ARMOUR LABORATORIES]

Synthesis of Compounds Related to Thymine. II. Effect of Thymine Antagonists on the Biosynthesis of DNA

BY THOMAS J. BARDOS, GEORGIA M. LEVIN, ROSS R. HERR AND HARRY L. GORDON

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The patterns of deoxyribonucleic acid biosynthesis have been studied in *Lactobacillus leichmannii* and *Lactobacillus arabinosus*. The modes of action of various metabolic antagonists, particularly 5-bromouracil and its nucleosides, are discussed. The systems described are used to study the biological action of three new thymine antagonists: 5-sulfur-substituted uracils.

In the course of a synthetic program designed to obtain compounds which would inhibit specific steps of nucleic acid biosynthesis, we have prepared several new structural analogs of thymine. The synthesis of three of these compounds, 5-mercaptouracil, 5-uracilyl disulfide and uracil-5-isothiouronium chloride was recently reported.[1]

For a systematic evaluation of the biological actions of our compounds, variations of the "inhibition analysis"[2] technique were used. Two microbiological systems were selected to represent two different patterns of nucleic acid biosynthesis. The two species, *Lactobacillus leichmannii* 313 and *Lactobacillus arabinosus*, were grown under standard conditions which were chosen to make specific steps of nucleic acid biosynthesis the "growth limiting reactions" of the systems. The new compounds were tested in both systems for their ability to inhibit the growth of these organisms and also to determine the reversibility of their growth inhibitory action by a number of metabolites, intermediates, and catalytic factors presumably involved in the biosynthesis of nucleic acids. However, to interpret our results, it was necessary to obtain additional information on the metabolic patterns of these organisms by studying the action of known inhibitors under the defined conditions of our systems.[3]

(1) T. J. Bardos, R. R. Herr and T. Enkoji, THIS JOURNAL, **77**, 960 (1955).

(2) W. Shive, *Ann. N. Y. Acad.*, **52**, 1212 (1950).

(3) For comparison with earlier work in this field, the authors wish to refer the reader especially to the papers of Hitchings and his collaborators on their "*Lactobacillus casei* model"; G. M. Hitchings, G. B. Elion, E. A. Falco, P. B. Russell, M. S. Sherwood and H. Vanderwerff, *J. Biol. Chem.*, **183**, 1 (1950); *Ann. N. Y. Acad.*, **52**, 1318 (1950).

Results and Discussion

Use of the technique of "inhibition analysis" can provide information useful in the understanding of biochemical interrelationships in given microbiological systems. However, evidence provided by this method cannot be considered as unequivocal. With this reservation in mind, and after comparing our results with those of other investigators using various lactobacilli, we wish to propose the hypothetical scheme presented in Fig. 1 for the biosynthesis of deoxyribosides, and use this as a basis for the subsequent discussion of our experimental results.

The medium used in our *L. leichmannii* experiments is composed so that the folic acid and vitamin B_{12} concentrations are fixed at levels just sufficient for maximal growth in 16 hours. The other metabolites, including the purines and uracil, are present in excess. Consequently, folic acid and vitamin B_{12} are the limiting growth factors in this system and, under the given conditions, the biosynthesis of the thymidine component of DNA appears to be the growth-rate limiting reaction.[4] In this biosynthesis both folic acid and vitamin B_{12} are involved as catalytic factors.[5] We found that folic acid can be replaced by folinic acid; the synthetic

(4) The terms "limiting reaction," "competitive," and "noncompetitive" inhibition, "product-effect," "inhibition index," etc., used by us in the discussion are defined by Shive in ref. 2 and in R. J. Williams, R. E. Eakin, E. Beerstecher and W. Shive, "The Biochemistry of B-Vitamins," Reinhold Publ. Corp., New York, N. Y., 1950, pp. 443–480.

(5) W. Shive, J. M. Ravel and W. M. Hardin, *J. Biol. Chem.*, **176**, 991 (1948).