ionized. The latter reaction was investigated in the pH range 6.46 to 7.45; the small ionization corrections were estimated using +800 and +10,800 cal. per mole for the second ionization heats of phosphoric acid[4] and leucine,[5] respectively, and pK_2' 9.60 for the second ionization of leucine.[5]

Results

The experimental results are summarized in Tables I and II. The notation adopted in previous work[2,3] has been followed. The rate constants given for each reaction have no absolute significance since it is known that the enzymes used were not highly purified preparations.

TABLE I

THE HYDROLYSIS OF GLYCYL-L-PHENYLALANYLAMIDE BY CATHEPSIN C AT 25°

Buffer concentration, 0.05 M; ionic strength, 0.3 M; P, sodium phosphate, A, sodium acetate

pH	Initial substrate concn., mole per l., × 10^3	Enzyme concn., mg. prot. nitrogen per ml.	Cysteine concn., moles per l., × 10^3	k, min.$^{-1}$	$-\Delta H_h$, cal. per mole	Buffer
4.69	0.587	0.0387	1.90	0.072	5900	P + A
4.69	.587	.0387	1.90	.066	5880	P + A
4.85	.453	.116	1.92	.088	6285	A
4.85	.439	.116	1.92	.086	6285	A
5.09	.453	.116	1.93	.107	6300	P
5.11	.694	.116	2.75	.137	6780	P
5.11	.453	.116	1.93	.115	6300	P
5.65	.342	.116	1.90	.095	5970	P
5.65	.342	.116	1.90	.093	6260	P
				Mean	6220 ± 150	

TABLE II

THE HYDROLYSIS OF CARBOBENZOXYGLYCYL-L-LEUCINE BY CARBOXYPEPTIDASE AT 25°

Sodium phosphate buffer, 0.05 M; ionic strength, 0.4 M

pH	Init. substrate concn., moles per l., × 10^3	Enzyme concn., mg. prot. nitrogen per ml.	$-\Delta H_{obsd.}$ cal. per mole	$-\alpha(\Delta H_i - \Delta H_p)$, cal. per mole	$-\Delta H_h$, cal. per mole	k_2/K_m
6.46	1.63	0.0321	2015	7	2022	0.89
6.46	1.63	.0321	2115	7	2122	1.01
6.77	1.49	.0391	2090	15	2105	1.03
6.77	1.49	.0391	2060	15	2075	0.99
6.81	2.35	.0414	2040	16	2056	1.34
6.81	2.35	.0414	2030	16	2046	1.27
7.24	1.87	.0413	2180	44	2224	1.92
7.24	1.87	.0413	2110	44	2154	1.85
7.44	1.14	.0413	2100	69	2169	2.21
7.45	1.14	.0321	2060	70	2130	2.55
				Mean	2110 ± 50	

Table III lists the heats of hydrolysis for the five compounds studied by our method. These values refer in each case to the formation of fully charged products, according to the equation

$$RCONHR' + H_2O = RCOO^- + {}^+NH_3R'$$

The data are as yet not sufficiently extensive to permit tracing in detail the relation between peptide structure and heat of hydrolysis. However, it is evident that the amide hydrolyses are considerably more exothermic than the peptide hydrolyses. It should be noted that, since the heat of ionization of the ammonium ion is about 2000 cal. per mole more positive than the heat of the second ionization of most amino acids, the heat of hydrolysis of an amide bond to give *uncharged* products would be more nearly equal to that of the peptide bond to give *uncharged* products. A small structural change, such as replacement of the isobutyl side chain of leucine by the benzyl side chain of phenylalanine, produces a small but definite change in the heat of hydrolysis. It is perhaps significant that the smallest heat is observed in the hydrolysis of the neutral molecule benzoyltyrosylglycinamide; presumably this value will be found to be approximately characteristic of the hydrolysis of the majority of the peptide bonds in a protein which are located at considerable distances from charged groups.

TABLE III

HEATS OF HYDROLYSIS OF SYNTHETIC PEPTIDES AT 25°

Substrate	Bond hydrolyzed	$-\Delta H_h$, cal. per mole
Benzoyl-L-tyrosinamide	Amide	5840 ± 220
Benzoyl-L-tyrosylglycinamide	Tyrosine–glycine	1550 ± 100
Carbobenzoxyglycyl-L-leucine	Glycine–leucine	2110 ± 50
Carbobenzoxyglycyl-L-phenylalanine	Glycine–phenylalanine	2550 ± 50
Glycyl-L-phenylalanylamide	Amide	6220 ± 150

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(4) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 2nd Ed., Reinhold Publ. Corp., New York, N. Y., 1950, p. 514.

(5) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides." Reinhold Publ. Corp., New York, N. Y., 1943, pp. 80 and 84.

Antimetabolites of Uridine with Two Structural Alterations[1]

BY DONALD W. VISSER, GERALD BARRON AND RICHARD BELTZ

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A number of 5-substituted uridine derivatives competitively inhibit the growth response of *Neurospora* 1298 produced by uracil, uridine or cytidine[2,3,4] and several of these compounds also inhibit the propagation of Theilers GD VII encephalomyelitis virus *in vitro*.[5] Substitution of the hydrogen in the 3-position of uridine produces a similar antimetabolite, 3-methyluridine.[3,4] It was of interest, therefore, to prepare nucleosides which are substituted in both the 3- and 5-positions and compare the biological activity of these compounds with the corresponding derivatives having a single structural alteration. The new compounds are of interest not only because of their structural similarity to uridine and cytidine, which are utilized for nucleic acid biosynthesis, but also may be of value

(1) This work was aided by a grant from Research Corporation. Presented, in part, before the Biochemistry Division, at the April, 1952, Meeting of the American Chemical Society at Milwaukee, Wisconsin.

(2) T. K. Fukuhara and D. W. Visser, *J. Biol. Chem.*, **190**, 95 (1951).

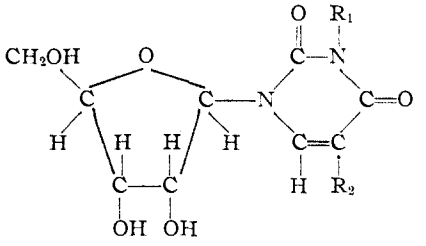
(3) M. Roberts and D. W. Visser, THIS JOURNAL, **74**, 668 (1952).

(4) M. Roberts and D. W. Visser, *J. Biol. Chem.*, **194**, 695 (1952).

(5) D. W. Visser, D. Langenborg and H. E. Pearson, *Proc. Soc. Expt. Biol. Med.*, **79**, 571 (1952).

for extending knowledge concerning the relationship between structure and antimetabolite activity.

The doubly substituted nucleosides were prepared by prior methylation of uridine according to a modified procedure of Levene and Tipson,[6] and the hydrogen on carbon atom 5 of the resulting 3-methyluridine was substituted by a bromine (I), chlorine (II) or an amino group (III) by procedures similar to those described by Roberts and Visser.[3]



No.	R_1	R_2	Name, 3-methyl-5-
I	CH_3	Br	Bromouridine
II	CH_3	Cl	Chlorouridine
III	CH_3	NH_2	Aminouridine

Experimental[7]

3-Methyluridine.—Tritylation of uridine was eliminated from the procedure reported by Levene and Tipson.[6] Dry uridine (7.0 g., 0.029 mole) was dissolved slowly in 100 ml. of freshly-distilled acetic anhydride. The cloudy solution was allowed to stand overnight at room temperature, and then slowly concentrated at reduced pressure (4 mm.) at 20–25° to an amber sirup (about two days). The product crystallized into a solid cake upon seeding. The cake was washed with absolute ethanol and recrystallized from boiling absolute alcohol (9.5 g., 90%).

The triacetyluridine (19 g., 0.051 mole) was dissolved in a minimum amount of hot ethylene dichloride. The solution was cooled to 0°, and 5.3 g. of diazomethane in ether was added slowly with shaking. The solution, in a flask fitted with a stopper containing a drying tube, was allowed to stand overnight at room temperature. The yellow solution was taken to dryness under reduced pressure (4 mm.) at room temperature. Absolute methyl alcohol (50 ml.) was added and the solution was again taken to dryness at reduced pressure.

The residue, a white semi-crystalline product, was dissolved in a 5% solution of dry HCl in dry methyl alcohol (2.6 g. HCl/500 ml. methyl alcohol), refluxed slowly for 10 minutes, and left to stand overnight. The solution was then taken to dryness at reduced pressure (4 mm.) and room temperature. Methyl alcohol (10 ml.) was added and the solvent was again removed to eliminate most of the excess hydrochloric acid.

The semi-crystalline amber product was taken up in a minimum amount of cold distilled water, filtered, and passed through Amberlite IRA-400 (about 5 g.). The effluent (*p*H 5–7) was decolorized with carbon and lyophilized. The white residue was dissolved in an equal mixture of methyl alcohol and ethyl acetate, and crystallization induced by the addition of ethyl ether to opalescence. It was found that the compound crystallized best in the cold from a concentrated solution. The solution yielded 9.8 g. (74%) of white needles, melting at 122–123°. Levene and Tipson[6] reported a m.p. of 108–110° for 3-methyluridine (both compounds give an identical analysis). The lower melting compound has also been obtained in this Laboratory by the procedure outlined above. However, attempts to interconvert these compounds were not successful.

3-Methyl-5-bromouridine (I).—High-melting 3-methyluridine (2.6 g., 0.010 mole) was dissolved in water and bromine water was added at 5° until the solution remained colored. Air was then bubbled through the solution to remove excess bromine and the colorless solution was lyophilized.

The sticky product was refluxed with absolute ethyl alcohol for two hours, and concentrated to a sirup on a water-bath. The product which crystallized upon cooling was recrystallized from absolute ethyl alcohol. The yield was 3.10 g. (91%) of a white crystalline compound, melting at 164–164.5°. *Anal.* Calcd. for $C_{10}H_{13}O_6N_2Br$: C, 35.63; H, 3.87; N, 8.31. Found: C, 35.99; H, 3.77; N, 8.54.

3-Methyl-5-chlorouridine (II).—3-Methyluridine (750 mg., 0.0029 mole) was suspended in 45 ml. of dry acetic acid and a 10% molar excess (0.32 g., 0.0045 mole) of dry chlorine dissolved in cold anhydrous carbon tetrachloride was added at room temperature and the resulting cloudy solution was allowed to stand overnight. After removal of solvent and acid the product was dissolved in a 1.0% solution of anhydrous HCl (0.44 g. in 44 ml.) in methyl alcohol. The solution was allowed to stand for two to five days. The acid was removed in the same manner as in the preparation of 3-methyluridine and the product crystallized from methyl alcohol. The crystallization which was favored by high concentration and cold yielded 300 mg. (35.3%) of white crystals melting at 158–159°. *Anal.* Calcd. for $C_{10}H_{13}O_6N_2Cl$: C, 41.02; N, 9.57; H, 4.48. Found: C, 40.93; N, 9.35; H, 4.85.

3-Methyl-5-aminouridine (III).—3-Methyl-5-bromouridine (1 g., 0.003 mole) was suspended in 30 ml. of absolute ethyl alcohol in a stainless steel bomb tube. The tube was cooled in a Dry Ice–acetone-bath and 8 ml. of liquid ammonia was added. The tube was sealed and allowed to come to room temperature and then heated at 55° for six days. The bomb was cooled in a Dry Ice–acetone mixture, opened, and the contents poured out. The ammonia and alcohol were removed from the amber solution under reduced pressure (aspirator) at room temperature. The product was taken up in a minimum of water and passed through 5 g. of IRA-120 ion exchange resin. The column was washed with 3 liters of water and the product was eluted with 500 ml. of 4 *N* aqueous ammonia. The ammonia was removed at reduced pressure and the solution lyophilized. The crystalline product was dissolved in a minimum amount of boiling ethyl alcohol, filtered, and allowed to crystallize in the cold. The yield was 500 mg. of fine, amber-tinted, semi-crystalline material melting at 159–168°. After crystallizations from absolute ethanol, 400 mg. (49%) of a white product was obtained, melting at 166–167°. *Anal.* Calcd. for $C_{10}H_{15}O_6N_3$: C, 43.96; N, 15.38, H, 5.53. Found: C, 44.24; N, 15.49; H, 5.40.

Microbiological.—The 3,5-substituted nucleosides were tested for growth-promoting or growth-inhibiting properties with a pyrimidine-requiring mutant of *Neurospora*, 1298, using a modification[8] of the basal medium of Horowitz and Beadle.[9] The mutant was incubated at 25° for three days in 50-ml. erlenmeyer flasks, each containing a total volume of 10 ml. of liquid medium.

The mutant was grown in the presence of given amounts of uracil, uridine or cytidine as the pyrimidine requirement and varying amounts of the 3,5-substituted nucleoside. The inhibition indices, the molar ratio of antimetabolite to metabolite which gives half-maximum growth, are summarized in Table I.

TABLE I

Inhibition indices[a]; *Neurospora* 1298

Antimetabolite, -uridine	Metabolite Uridine	Metabolite Cytidine	Uracil
3-Methyl-5-bromo-	7.3[b]	1.2[b]	
3-Methyl-5-chloro-	6.4[b]	1.7[b]	
3-Methyl-5-amino-		9.1[b]	No inhib.[c]
3-Methyl-	4.0	0.47	No inhib.[c]
5-Chloro-	3.0	0.55	No inhib[c]
5-Amino-	14.0	3.0	0.2[d]

[a] Moles of antimetabolite per mole of metabolite giving 50% of maximum growth. Grown for three days at 25° in 50-ml. erlenmeyer flasks with a total volume of 10 ml. of medium. [b] Inhibition is competitive from a concentration range of 0.6 to 0.9 mg. substrate in 10 ml. of medium. [c] To 3.2 moles of antimetabolite per mole of metabolite. [d] Inhibition is not competitive.

(6) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **104**, 385 (1934).

(7) All melting points were taken on a Fisher–Johns melting point apparatus and are uncorrected.

(8) F. J. Ryan and E. Brand, *J. Biol. Chem.*, **154**, 161 (1944).

(9) N. H. Horowitz and G. W. Beadle, *ibid.*, **150**, 325 (1943).

Discussion

It is apparent that the 3,5-substituted nucleosides are less effective as antimetabolites than are the nucleosides in which only one of these structural changes exists. For example, 3-methyluridine or 5-chlorouridine have an inhibition index of approximately 0.5 when cytidine provides the pyrimidine requirement. When both structural changes are made on the same molecule, 3-methyl-5-chlorouridine, the inhibition index against cytidine is increased by a factor of about 3. A similar increase in the inhibition index obtained with the doubly substituted nucleosides is observed whether uridine, cytidine, or uracil provide the pyrimidine requirement. It is of interest to note that a methyl group in the 3-position of uridine decreases the activity of the antimetabolite whether the substituent on the 5-carbon is nucleophilic or electrophilic.

The results agree with the observation of Woolley and Pringle[10] who have demonstrated that as the structural difference between metabolite and analog increases, the degree of inhibition usually decreases. However, over the range of substrate concentration tested, the doubly substituted nucleosides retain their ability to inhibit in a competitive manner.

(10) D. W. Woolley and A. Pringle, *J. Biol. Chem.*, **194**, 729 (1952).

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COMMUNICATIONS TO THE EDITOR

IONIC INHIBITION OF GROWTH IN LACTOBACILLUS LEICHMANNII 313 AND ITS REVERSAL WITH VITAMIN B_{12}

Sir:

Vitamin B_{12}, as a growth factor for *Lactobacillus Leichmannii* 313, can be replaced by thymidine[1] or other desoxyribosides.[2] It has been suggested[1,3] that vitamin B_{12} might function as a catalyst (coenzyme) in the formation of desoxyribosides. The experimental data presented here seem to offer some indirect evidence for the existence of a vitamin B_{12}-enzyme.

We found that slightly hypertonic concentrations of various inorganic salts inhibit the growth of *L. Leichmannii* 313, in a basal medium[4] supplemented with just sufficient (0.1 mγ per 5 ml.) vitamin B_{12} to allow full growth (in the absence of the salts). This inhibition can be reversed with an added excess of vitamin B_{12}. When the salt concentration is increased, the vitamin B_{12} requirement sharply increases. Through a narrow salt concentration range, which we will term the "reversible range" (*e.g.*, in the case of NaCl from 1.1 to 1.7%), the inhibition can be fully reversed by increasing the vitamin B_{12} level from the initial 0.1 mγ up to about 25 mγ (per 5 ml.); above this range, only partial reversal can be obtained during a standard, 16 hour, incubation period. Thymidine, throughout the "reversible range" supports full growth at slightly increasing (5–10 γ per 5 ml.) levels; above the "reversible range," the maximum growth response obtained with thymidine is the same as with excess vitamin B_{12} (see Table I).

TABLE I

Salt	Concentration %	Concentration M[a]	μ[b]	$(B_{12})_{1/2}$ max.[c]	Thymidine[d]
None	...	...	...	0.025[e]	1.8
NaCl	1.4	0.239	0.239	1.0	
	1.5	.256	.256	1.5	2.0
	1.7	.291	.291	6.0	2.5
	1.9	.325	.325	(25.0)[f]	(2.5)[f]
KCl	1.62	.217	.217	0.50	1.8
	1.88	.252	.252	1.58	1.8
	2.13	.285	.285	7.5	2.0
	2.37	.318	.318	(25)[f]	(2.2)[f]
NH_4Cl	1.2	.224	.224	0.45	
	1.4	.262	.262	2.30	2.5
	1.6	.299	.299	5.0	
	1.8	.336	.336	(20)[f]	
K_2SO_4	1.6	.092	.276	0.12	1.8
	1.8	.103	.309	0.40	1.8
	2.0	.115	.365	0.60	
	2.3	.132	.406	1.20	
	2.6	.149	.447	5.0	
	2.9	.166	.498	(15)[f]	
$MgCl_2 \cdot 6H_2O$	1.0	.049	.196	0.14	
	1.2	.059	.236	0.30	
	1.4	.069	.273	1.15	
$CaCl_2$	0.8	.073	.292	3.8	
	1.2	.109	.436	(25)[f]	

[a] Gram moles per liter. [b] Ionic strength, $\mu = {}^1/_2\Sigma cv^2$, where c = gram ions per liter; v = valence, for each ion. [c] mγ per 5 ml.; amount of additional (in excess of 0.1) vitamin B_{12} needed for half maximum growth. [d] γ per 5 ml.; required for half maximum growth in vitamin B_{12}-free media. (We are indebted to Dr. W. Shive for a small sample of this substance.) [e] mγ per 5 ml. basal medium (vitamin B_{12} standard curve). [f] Salt concentration above "reversible range"; only partial growth obtained.

(1) W. Shive, J. M. Ravel and R. E. Eakin, THIS JOURNAL, **70**, 2614 (1948).

(2) E. Kitay, W. S. McNutt and E. E. Snell, *J. Biol. Chem.*, **177**, 993 (1949).

(3) E. Kitay, W. S. McNutt and E. E. Snell, *J. Bact.*, **59**, 727 (1950).

(4) Per 100 ml.: acid-hydrolyzed casein, 0.5 g.; L-cysteine hydrochloride, 10 mg.; DL-tryptophan, 20 mg.; L-asparagine, 10 mg.; DL-alanine, 20 mg.; adenine sulfate, 1 mg.; guanine hydrochloride, 1 mg.; uracil, 1 mg.; xanthine, 1 mg.; thiamin hydrochloride, 100 γ; pyridoxine, 200 γ; pyridoxamine, 60 γ; pyridoxal, 60 γ; calcium pantothenate, 100 γ; niacin, 200 γ; PABA, 20 γ; biotin, 0.2 γ; folic acid, 0.4 γ; riboflavin, 100 γ; ascorbic acid, 0.2 g.; dextrose, 2.0 g.; tween 80, 100 mg.; salts A, 1 ml.; salts B, 1 ml.; sodium acetate, 0.5 g. Incubation, 16 hours at 37°. Five ml. in each tube.