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).

(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.

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]	
$MgCl_2\cdot6H_2O$	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.

Table I gives the amounts of vitamin B_{12} required for half maximum growth at various salt concentrations. *The logarithm of the vitamin B_{12} requirement appears to be a linear function of the ionic strength of the salt solutions*

$$\log (B_{12})_{1/2\ max.} = a + b/\mu$$

This equation seems to apply well within the "reversible range" and the values of the constants a and b, respectively, are not too far apart for most of the salts examined.

It is possible to arrive theoretically to a similar functional relationship between $(B_{12})_{1/2\ max.}$ and μ from simple kinetic equations, if we make two basic assumptions: first, vitamin B_{12} combines with a protein apoenzyme (Ea) to give the enzyme $B_{12}Ea$: $B_{12} + Ea \rightleftarrows B_{12}Ea$; second, the available concentration of Ea is controlled by the ionic strength of the salt solutions in accordance with Cohn's "salting-out" equation for proteins.[5,6] These two assumptions allow the derivation of a theoretical equation which has the same form as the experimental formula. The derivation itself, together with a critical appraisal of such interpretation of our data, will be presented elsewhere.

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(6) M. Ingram, *Proc. Roy. Soc., Ser. B*, **134**, 181 (1951).

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ASCORBIC ACID DEFICIENCY AND CHOLESTEROL SYNTHESIS[1]

Sir:

In continuing studies of chemical changes characteristic of or regulated by ascorbic acid[2,3] and related metabolites,[4] we have recently observed a relationship to steroid metabolism that is of considerable interest. Although 1-C^{14}-labeled ascorbic acid is not appreciably incorporated into cholesterol, the vitamin does exert a marked effect upon the conversion of acetate-1-C^{14} to cholesterol and other steroids in guinea pigs. Preliminary findings showed that severely scorbutic guinea pigs, compared with normal animals fed *ad lib.*, incorporated 6 times as much C^{14} from acetate-1-C^{14} into cholesterol isolated from adrenals.

Guinea pigs of comparable age (10–12 weeks) and size (350–400 g.), on a vitamin C- and cholesterol-free chow diet showed the following values (3 animals per group) for specific activities in purified adrenal and liver cholesterol, respectively, four hours after receiving the last of three intraperitoneal injections of labeled sodium acetate (1 mg., 2.68×10^7 c.p.m./mg. each at 9 hour intervals): normal, fed *ad lib.*, 100 and 80; mild scurvy (15–20 days depletion), 170 and 75 (pair-fed controls, 150 and 80); severe scurvy (21–28 days depletion), 600 and 145 (pair-fed controls, 195 and 90).

Initial cholesterol fractions showed the presence of small quantities of similar C^{14}-labeled components but there was only a slight change in activity of the cholesterol after purification by precipitation of the digitonide, dibromination[5] and recrystallization. The observed changes in C^{14} content were not accompanied by comparable changes in total cholesterol present in the tissues[6] but they were sufficient to indicate changes in the C^{14}-content of other sterols.

(1) This work was supported in part by grants from the Nutrition Foundation, Inc., and the Division of Research Grants, U. S. Public Health Service.

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(6) K. Guggenheim and R. E. Olson, *J. Nutrition*, **48**, 345 (1952).

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ENZYMATIC PHOSPHORYLATION OF NUCLEOSIDES BY PHOSPHATE TRANSFER

Sir:

We have found a phosphatase preparation, obtained by the fractionation with ammonium sulfate of Merck malt diastase, which is able to phosphorylate ribose and desoxyribose nucleosides in the presence of sodium phenylphosphate. The reaction is dependent on the concentrations of both nucleoside and phenylphosphate. The pH activity curves for transphosphorylation and dephosphorylation have the same shape, with an optimum around pH 5.2. Both reactions are partially inhibited by inorganic phosphate to the same extent.

The organic phosphates formed were separated by paper chromatography with aqueous isobutyric acid buffered with ammonium isobutyrate as the solvent.[1] Their R_F values were identical with those of the corresponding nucleotides.

In a large-scale experiment, 166 μ moles of ribocytidine was incubated, in a total volume of 4 ml., with 800 μ moles of phenylphosphate and 8 mg. of enzyme in 0.1 M acetate buffer of pH 5 for 87 hours at 30°. At this stage, 80% of the phosphate donor were split and 17 μ moles of cytidylic acid (10.2% of the nucleoside) were formed. The cytidylic acid fraction, isolated by ion-exchange chromatography,[2] contained equimolar quantities of organic phosphorus and of nucleoside (determined spectrophotometrically) and was completely dephosphorylated by the 5-nucleotidase of rattlesnake venom which, under the conditions used, failed to attack commercial cytidylic acid consisting, presumably, of a mixture of the 2′- and 3′-nucleotides. This evidence tends to indicate that the 5′-nucleotide had been produced.

All nucleosides tested could thus be phosphorylated. Preliminary results, listed in Table I, apparently show that, under identical conditions, desoxyribonucleosides[3] are phosphorylated with greater ease than the corresponding ribosides.

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(2) W. E. Cohn and E. Volkin, *Nature*, **167**, 483 (1951).

(3) Uracil desoxyriboside was obtained through the courtesy of Prof. A. R. Todd.