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).

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION
SCHOOL OF MEDICINE, AND
LABORATORIES OF THE ALLAN HANCOCK FOUNDATION
UNIVERSITY OF SOUTHERN CALIFORNIA
LOS ANGELES, CALIFORNIA

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.

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.

(5) E. J. Cohn, *Physiol. Rev.*, **5**, 349 (1935); *Ann. Rev. Biochem.*, **4**, 93 (1935).

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

THE ARMOUR LABORATORIES THOMAS J. BARDOS
CHICAGO 9, ILLINOIS HARRY L. GORDON

RECEIVED MARCH 18, 1953

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).

(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.

(2) L. L. Salomon, J. J. Burns and C. G. King, THIS JOURNAL, **74**, 5161 (1952).

(3) J. J. Burns, H. B. Burch and C. G. King, *J. Biol. Chem.*, **191**, 501 (1951).

(4) Hugh H. Horowitz and C. G. King, *ibid.*, **200**, 125 (1953).

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.

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

R. R. BECKER
H. B. BURCH
DEPARTMENT OF CHEMISTRY L. L. SALOMON
COLUMBIA UNIVERSITY T. A. VENKITASUBRAMANIAN
NEW YORK 27, NEW YORK C. G. KING

RECEIVED MARCH 23, 1953

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 *p*H activity curves for transphosphorylation and dephosphorylation have the same shape, with an optimum around *p*H 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 *p*H 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.

(1) B. Magasanik, E. Vischer, R. Doniger, D. Elson and E. Chargaff, *J. Biol. Chem.*, **186**, 37 (1950).

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

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

TABLE I

PHOSPHORYLATION OF NUCLEOSIDES BY MALT ENZYME

The solutions containing, per ml. of 0.1 *M* acetate buffer of *p*H 5, 40 μ moles of nucleoside, 200 μ moles of phenylphosphate and 2 mg. of enzyme were incubated at 30° for 30 hours, when about 55% of the phosphorus has become inorganic

Nitrogenous constituent	Extent of phosphorylation, as % of nucleoside	
	Riboside	Desoxyriboside
Hypoxanthine	5.0	8.9
Uracil	7.7	9.9
Cytosine	7.6	13
Thymine		9.4

The transphosphorylation system described here seems to be the first instance of a general enzymatic reaction leading to the formation of nucleotides from nucleosides. In some of its characteristics it resembles the transfer reactions studied by Axelrod[4] and Green and Meyerhof.[5]

This work was supported by research grants from the National Institutes of Health, U. S. Public Health Service, and the Rockefeller Foundation. One of us (G.B.) was aided by a Predoctorate Research Fellowship from the U. S. Public Health Service.

(4) B. Axelrod, *J. Biol. Chem.*, **172**, 1 (1948).
(5) H. Green and O. Meyerhof, *ibid.*, **197**, 347 (1952).

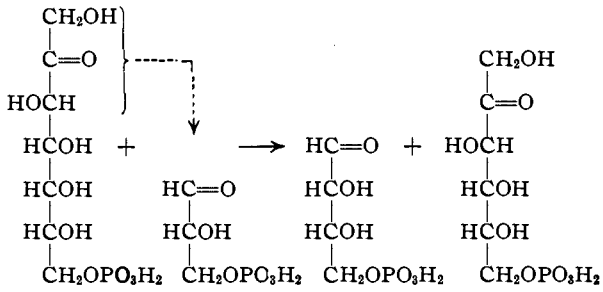
DEPARTMENT OF BIOCHEMISTRY
COLLEGE OF PHYSICIANS AND SURGEONS
COLUMBIA UNIVERSITY GEORGE BRAWERMAN
NEW YORK 32, N. Y. ERWIN CHARGAFF

RECEIVED MARCH 16, 1953

TRANSALDOLASE: THE FORMATION OF FRUCTOSE-6-PHOSPHATE FROM SEDOHEPTULOSE-7-PHOSPHATE

Sir:

Previous reports[1-3] have described the formation of ribulose-5-phosphate and sedoheptulose-7-phosphate as intermediates in the oxidation of hexose-monophosphate. This pathway is now established as a cyclic process by the finding that sedoheptulose-7-phosphate is converted to fructose-6-phosphate. Preparations from liver and yeast have been obtained which catalyze this reaction, but only in the presence of a source of triose phosphate (Table I). With a purified preparation from brewer's yeast the reaction is stoichiometric with respect to either sedoheptulose-7-phosphate or triose phosphate. The reaction mechanism has been clarified by the use of C^{14}-triose phosphate. Hexosemonophosphate produced from unlabeled sedoheptulose-7-phosphate and uniformly labeled triose phosphate (derived from fructose diphosphate) had a specific activity of 53,000 c.p.m. per micromole compared with 58,000 c.p.m. per micromole for the triose phosphate, indicating that three carbon atoms of the hexosemonophosphate were derived from triose phosphate. Degradation of the glucose obtained by hydrolysis of the hexose-monophosphate showed the label to be present only in carbon atoms 4, 5 and 6; carbon atoms 1, 2 and 3 which contained no radioactivity must have come from sedoheptulose-7-phosphate.[4] It is concluded from these results that the reaction mechanism involves a transfer of the dihydroxyacetone group from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate to form fructose-6-phosphate and erythrose-4-phosphate.



Since this enzyme catalyzes a transfer of aldol linkages, rather than their hydrolytic cleavage, it will be referred to as transaldolase.

The formation of fructose-6-phosphate as the initial reaction product is confirmed by the results in Table I, since little glucose-6-phosphate is formed in the absence of hexose phosphate isomerase.

TABLE I

THE FORMATION OF GLUCOSE-6-PHOSPHATE FROM SEDOHEPTULOSE-7-PHOSPHATE

The complete system contained 3.1×10^{-4} *M* sedoheptulose-7-phosphate, 3.3×10^{-4} *M* fructose diphosphate, 2.5×10^{-5} *M* triphosphopyridine nucleotide (TPN), 5.6 μg. of purified yeast transaldolase, 83 μg. of aldolase, 110 μg. of glucose-6-phosphate dehydrogenase and 340 micrograms of hexose phosphate isomerase, in 0.04 *M* triethanolamine buffer, *p*H 7.6. The total volume was 1.10 ml. and the temperature was 25°. The production of glucose-6-phosphate was followed spectrophotometrically by measuring the reduction of TPN in the presence of glucose-6-phosphate dehydrogenase. Hexose phosphate isomerase was a crude rabbit muscle ammonium sulfate fraction; all other enzymes were purified preparations.

System	Glucose-6-phosphate produced[a]
Complete system	4.7
Sedoheptulose-7-phosphate omitted	.0
Hexosediphosphate omitted	.0
Hexose phosphate isomerase omitted	.4
Transaldolase omitted	.4

[a] Millimicromoles per minute.

Preliminary evidence for a tetrose ester in the reaction mixture has been obtained with paper chromatography following hydrolysis with potato phosphatase. The chromatogram was developed with a 70–30 acetone–water mixture and sprayed with aniline phthalate. A fluorescent spot was observed with R_f = 0.65, identical with authentic erythrose.[5] However, since this solvent does not separate the aldo- and keto-tetroses, the nature of the tetrose remains to be determined.

The transfer of aldol linkages from one sugar to another, like the transfer of the ketol groups previ-

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(2) J. E. Seegmiller and B. L. Horecker, *ibid.*, **194**, 261 (1952).
(3) B. L. Horecker and P. Z. Smyrniotis, THIS JOURNAL, **74**, 2123 (1952).

(4) We are indebted to Dr. M. Gibbs of the Brookhaven National Laboratory for the degradation of the labeled glucose.
(5) Kindly furnished by Dr. H. S. Isbell of the National Bureau of Standards.

ously reported,[6,7] provides a new mechanism for the biological transformation of sugars. The formation of glucose from galactose[8] or ribose phosphate from xylose[9] may occur by such transfer or exchange reactions.

(6) B. L. Horecker and P. Z. Smyrniotis, THIS JOURNAL, **75**, 1009 (1953).
(7) E. Racker, G. de la Haba and I. G. Leder, *ibid.*, **75**, 1010 (1953).
(8) R. H. Caputto, L. F. Leloir, C. E. Cardini and A. C. Paladini, *J. Biol. Chem.*, **184**, 333 (1950).
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NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES
NATIONAL INSTITUTE OF HEALTH
PUBLIC HEALTH SERVICE
FEDERAL SECURITY AGENCY
BETHESDA 14, MD.
B. L. HORECKER
P. Z. SMYRNIOTIS

RECEIVED MARCH 14, 1953

THE ENZYMATIC SYNTHESIS OF N-ACETYLIMIDAZOLE

Sir:

Extracts of *Clostridium kluyveri* oxidize butyrate to acetyl phosphate (acetyl-P) and acetate in orthophosphate buffer[1] and to acetoacetate in the absence of orthophosphate (Pi).[2] In the presence of imidazole (Pi absent) a labile acetyl compound is formed. This compound has been tentatively identified as N-acetylimidazole (AcI) on the basis of comparative studies with the synthetic compound.[3] AcI and the enzymatic product are readily hydrolyzed at *p*H 7.0 (30°), but in aqueous solution they react preferentially with amino acids, alcohols, Pi and sulfhydryl compounds to give the corresponding acetyl derivatives and with neutral hydroxylamine to give acethydroxamic acid.

The acetylation of Pi (reaction 1) is of particular interest since it establishes the energy-rich nature of AcI.

$$\text{AcI} + \text{Pi} \rightleftharpoons \text{acetyl-P} + \text{Imidazole} \qquad (1)$$

Equimolar amounts of AcI and Pi (0.1 *M*, *p*H 7.0) react to give a 50% yield of acetyl-P (20 min., 30°). The free energy of hydrolysis of AcI is therefore at least as great as that of acetyl-P (*i.e.*, 12,000–15,000 cal.[4]).

Advantage has been taken of the strong absorption band of AcI at 235–255 mμ to demonstrate reversibility of reaction 1. Thus an increase in optical density at 245 mμ is observed when acetyl-P is incubated with imidazole (*p*H 7.0, 25°). The non-enzymatic reaction does not occur readily at low acetyl-P concentrations (0.01 *M*); however, in the presence of dialyzed extracts of *C. kluyveri* a rapid enzymatic acetylation of imidazole occurs. The enzymatic reaction may be followed spectrophotometrically, as above, or indirectly by measuring the decrease in acetyl-P when incubated with imidazole in the presence of enzyme.

A partially purified imidazole acetylase (IA) obtained by fractionation of the bacterial extracts will not catalyze the acetylation of imidazole with acetyl-P unless Coenzyme A (CoA) and phosphotransacetylase (PTA)[5] are added (Table I).

TABLE I

REQUIREMENTS FOR THE ENZYMATIC ACETYLATION OF IMIDAZOLE

The complete system contained acetyl-P, 5 micromoles; cysteine, 50 micromoles; imidazole, 100 micromoles; triethanolamine·HCl buffer, 100 micromoles; CoA, 0.05 micromole; PTA, 9 units; IA, 1.5 mg. protein. The final volume was 0.5 ml. (*p*H 7.0). Samples were incubated at 30° for 20 min.

	Δ Acetyl-P[a]
Complete system	2.6
Complete system − Imidazole	0
Complete system − CoA	0.7
Complete system − PTA	.3
Complete system − IA	.2
Complete system − CoA + PTA	0

[a] Amounts in micromoles.

The requirements for CoA and PTA in addition to IA suggest that the following reactions are involved.

$$\text{Acetyl-P} + \text{CoA} \overset{\text{PTA}}{\rightleftharpoons} \text{Acetyl CoA} + \text{Pi} \qquad (2)$$

$$\text{Acetyl-CoA} + \text{Imidazole} \overset{\text{IA}}{\rightleftharpoons} \text{AcI} + \text{CoA} \qquad (3)$$

$$\text{AcI} + \text{Cysteine} \xrightarrow{\text{Non-enzymatic}} \text{Imidazole} + \text{N-acetylcysteine (and/or 2-methyl-4-carboxythiazoline)} \qquad (4)$$

$$\text{Acetyl-P} + \text{cysteine} \longrightarrow \text{N-acetylcysteine (thiazoline ?)} \qquad (5)$$

Cysteine is used in the test system as the ultimate acetyl acceptor since the N-acetylcysteine (or the thiazoline derivative which may be formed by ring closure) produced does not form a hydroxamic acid under the conditions used.[6] Thus the reaction can be followed by measuring the decrease in acetyl-P by the hydroxamic acid method.[7] The relatively slow direct non-enzymatic reaction between acetyl CoA and cysteine[6] does not occur to a significant extent under these experimental conditions (*p*H 7.0, low CoA concentration). Substitution of glutathione for cysteine in the test system leads to the accumulation of S-acetyl glutathione which was identified as previously described.[6]

The enzymatic formation of AcI appears significant for a theory of acetyl transfer at the high energy level in which imidazole may serve as a model compound in reactions that normally involve a naturally occurring imidazole derivative or related compound (possibly a coenzyme). In terms of the mechanism of enzyme-action it is suggested that the imidazole moieties of the histidine components of proteins may be implicated as acyl carriers in acyl-transfer reactions.

NATIONAL HEART INSTITUTE
NATIONAL INSTITUTES OF HEALTH
PUBLIC HEALTH SERVICE
FEDERAL SECURITY AGENCY
BETHESDA 14 MARYLAND
E. R. STADTMAN
F. H. WHITE, JR.

RECEIVED FEBRUARY 26, 1953

(1) E. R. Stadtman and H. A. Barker, *J. Biol. Chem.*, **180**, 1095 (1949).
(2) E. P. Kennedy and H. A. Barker, *ibid.*, **191**, 419 (1951).
(3) J. H. Boyer, THIS JOURNAL, **74**, 6274 (1952).
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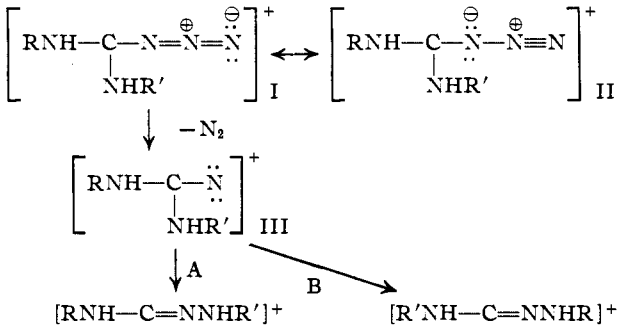
(6) E. Stadtman, *ibid.*, **196**, 535 (1952).
(7) F. Lipmann and L. C. Tuttle, *ibid.*, **158**, 505 (1945).

MIGRATION OF NITROGEN IN THE SCHMIDT REACTION

Sir:

From a consideration of the mechanism proposed for the isomerization of substituted 5-aminotetrazoles[1,2] together with a study of the products formed by the acid induced degradation of 5-substituted tetrazoles,[3] we have concluded that guanyl azides can undergo a Curtius or Schmidt type reaction.



Under the conditions used the carbodiimides hydrolyze to carbon dioxide, an amine and a hydrazine. The rearrangement of III involves an interchange in which the electrons are transported to the electronically deficient nitrogen by a nitrogen atom rather than a carbon atom. This has not been previously observed and affords another opportunity for studying the competitive migration aptitudes of groups. The degradation of 5-hydrazinotetrazole[4] in concentrated hydrochloric acid at 170° is an example. Opening of the tetrazole ring[2] yields a guanyl azide where R = NH_2 and R′ = H. Since ammonia is absent in the products, the rearrangement must occur almost exclusively by route A; *i.e.*, the amino group migrates in preference to the hydrazino group. We have found that 5-phenylaminotetrazole (IV) degrades largely to aniline and hydrazine (route A, where R = C_6H_5 and R′ = H): A solution of 0.5 g. of IV in 10 ml. of 85% phosphoric acid was heated at 190–200° for 2.5 hours, cooled, diluted with 10 ml. of water, refluxed for 1.5 hours, re-cooled, partially neutralized, and treated with benzaldehyde. Benzalazine (0.51 g. 79%; m.p. 92–93°) was removed and the solution was made alkaline. Benzalaniline (0.38 g., 68%; m.p. 46–49°) was recovered.

The Schmidt reaction should be capable of extension to the ammonocarbonic acids[5] provided that one of the contributing forms is a carbonium ion. With guanidine the product should be aminoguanidine and experimentally the latter has been recovered in about 1% yield. Ten grams of sodium azide was added portionwise during 2 hours to an agitated slurry of 10.8 g. of guanidine sulfate, 35 ml. of 96% sulfuric acid and 150 ml. of benzene at 25–28°. The temperature was next raised to and held at 40–50° for 4 hours. After the benzene layer was decanted, the acid layer was diluted with water, partially neutralized, shaken with benzaldehyde, neutralized to *p*H 9, and cooled to 5°. The hydrazone was removed, washed with water, extracted with petroleum ether until free of benzalazine, and converted to a picrate (0.3 g., 1%) which alone and in admixture with an authentic sample of benzalaminoguanidine picrate melted at 252–254°. X-Ray powder patterns were identical. Under similar conditions hydrazine and guanidine sulfates do not react to give aminoguanidine.

This extension of the Schmidt reaction will be fully explored.

CHEMISTRY DIVISION EUGENE LIEBER
U. S. NAVAL ORDNANCE TEST STATION RONALD A. HENRY
CHINA LAKE, CALIFORNIA WILLIAM G. FINNEGAN

RECEIVED MARCH 6, 1953

(1) W. G. Finnegan, R. A. Henry and E. Lieber, submitted to *J. Org. Chem.*

(2) R. A. Henry, W. G. Finnegan and E. Lieber, accepted for presentation at the 123 National Meeting of the American Chemical Society, Los Angeles, California.

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THE CYCLIZATION OF SQUALENE IN CHOLESTEROL SYNTHESIS

Sir:

The hypothesis that the triterpenoid hydrocarbon squalene is an intermediate in the biological synthesis of cholesterol (I) has recently received direct experimental support.[1,2] It has further been shown[3] that acetic acid, the principal carbon source in cholesterol synthesis, is a precursor also for squalene. The squalene hypothesis can be harmonized with the hitherto known distribution of acetate carbon in cholesterol[4] if one assumes that (a) each isoprene unit of the hydrocarbon contains 3 methyl and 2 carboxyl carbons of acetate, arranged as shown: 

(o = acetate methyl, x = acetate carboxyl); and (b) that in the transformation to sterol, squalene cyclizes as suggested by Robinson[5] (*cf.* Fig. 1, A).

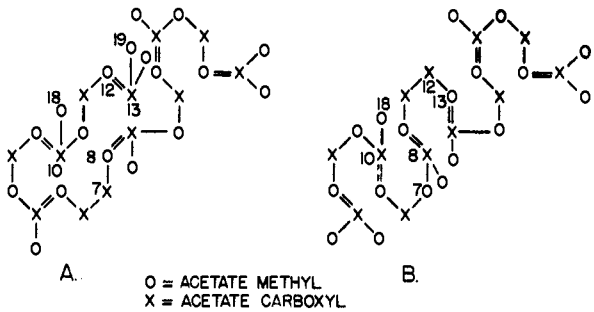


Fig. 1.

We now wish to propose the alternative mechanism shown in Fig. 1 B, as a more likely one to be involved in this transformation. Robinson's scheme provides for a conversion without rearrangement of the carbon skeleton of squalene. In mechanism B, on the other hand, one or more methyl migrations is necessary at some stage for the construction of the quaternary center at C_{13}. As is clear from Fig. 1, cyclization of squalene ac-

(1) R. G. Langdon and K. Bloch, THIS JOURNAL, **74**, 1869 (1952).

(2) R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **200**, 135 (1953).

(3) R. G. Langdon and K. Bloch, *ibid.*, **200**, 129 (1953).

(4) J. Würsch, R. L. Huang and K. Bloch, *ibid.*, **195**, 439 (1952).

(5) R. Robinson, *J. Soc. Chem. Ind.*, **53**, 1062 (1934).

cording to scheme B, instead of A, will alter the arrangement of acetate carbons in cholesterol only at the four following positions: C_7, C_8, C_{12} and C_{13}. Evidence for the origin of one of these crucial carbon atoms is presented in this communication. Epiandrosterone, obtained by degradation of labeled dihydrocholesterol,[4,6] was oxidized under the conditions used for C-methyl determination[7] to yield 1.7 moles of acetic acid (theory 2 moles). The acetic acid, which is derived from the angular methyl groups and the adjoining carbon atoms, was analyzed for C^{14} and degraded for separate assay of $C_{10} + C_{18}$ and $C_{18} + C_{19}$. The table shows that in cholesterol which had been synthesized biologically from methyl-labeled acetate, either C_{10} or C_{13}, in addition to the angular methyl groups, contains C^{14}. Since it is known from the work of

TABLE I

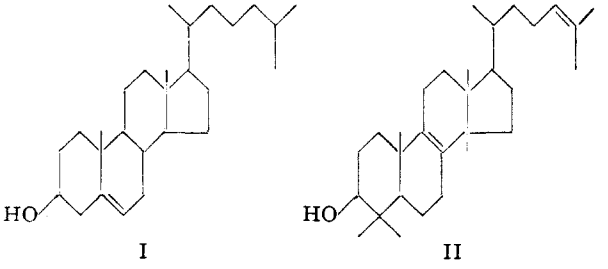
CHEMICAL DEGRADATION OF STEROID SYNTHESIZED FROM METHYL-LABELED ACETATE C^{14}, C.P.M.[a]

All values calculated for a ratio of 10 methyl to 9 carboxyl carbon atoms of acetate in epiandrosterone (*cf.* H. N. Little and K. Bloch, *J. Biol. Chem.*, 183, 33 (1950).

Products analyzed	Scheme A[b]	Scheme B[c]	Found
(1) Epiandrosterone	...	...	100
(2) Acetic acid from (1)	95	143	135
(3) Carboxyl-C from (2) $C_{10} + C_{13}$	0	95	105
(4) Methyl-C from (2) $C_{18} + C_{19}$	190	190	165

[a] As infinitely thick samples of $BaCO_3$. [b] Calcd. A: C_{18}, C_{19} derived biologically from acetate-methyl; C_{10}, C_{13} derived biologically from acetate-carboxyl. [c] Calcd. B: C_{18}, C_{19}, C_{13} derived biologically from acetate-methyl; C_{10} derived biologically from acetate-carboxyl.

Cornforth, *et al.*,[8] that C_{10} is derived from an acetate carboxyl, it can be concluded that the labeled carbon in the present case is C_{13}.[9] Now if C_{13} has its origin in a methyl group of acetic acid cyclization scheme A is untenable. Scheme B, which is consistent with earlier as well as with the present new isotopic data, provides also a particularly reasonable basis for the biosynthesis of the triterpenoid alcohol, lanosterol (II), whose remarkable resemblance to the sterols has recently been demonstrated.[10] Formation from squalene, accompanied by a methyl shift, would rationalize the fact that lanosterol is not constituted in accordance with the isoprene rule.



I II

(6) Isolated by Dr. Josef Würsch.

(7) R. Kuhn and F. L'Orsa, *Z. angew. Chem.*, **44**, 847 (1931).

(8) J. W. Cornforth, G. D. Hunter and G. Popjak, private communication.

(9) These arguments will not be valid in the very unlikely event that the angular methyl carbon C_{18} shifts quantitatively to another position during the course of the oxidation.

(10) W. Voser, M. V. Mijovic, H. Heusser, O. Jeger and L. Ruzicka, *Helv. Chim. Acta*, **35**, 2414 (1952).

CONVERSE LABORATORY
HARVARD UNIVERSITY
CAMBRIDGE, MASS. R. B. WOODWARD

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF CHICAGO
CHICAGO, ILL. KONRAD BLOCH

RECEIVED MARCH 11, 1953

ENZYMATIC REDUCTION OF COMPOUND E-21-ALDEHYDE TO COMPOUND E

Sir:

Δ^4-Pregnene-17α-ol-21-al-3,11,20-trione (Compound E-21-aldehyde) has been prepared from Δ^4-pregnene-17α,21-diol-3,11,20-trione (Compound E) by Rogers, *et al.*,[1] who noted that the aldehyde has approximately the same biological activity as the parent hormone. We believe that the observed activity of the aldehyde is due to its rapid reduction to Compound E *in vivo*, and wish to report the isolation of Compound E following the incubation of the aldehyde in a simple cell-free *in vitro* system.

Six grams of rat liver acetone powder was extracted with 60 ml. of Kreb's phosphosaline buffer *p*H 7.4 for 30 min. at room temp. The suspension was centrifuged, and the supernatant (48 ml.) added to 57.8 mg. of the aldehyde hydrate[2] in 68 ml. of the above buffer containing 100 mg. of dihydrodiphosphopyridine nucleotide. After 30 min. at 38° (gas phase air) the incubate was diluted with acetone, filtered, and the acetone removed *in vacuo*. The aqueous residue was diluted with water and extracted with ethyl acetate. After removal of the solvent, the extract was chromatographed on magnesium silicate Celite. Elution with ethyl acetate gave a single crystalline fraction which after recrystallization from methanol weighed 22 mg. It was identified as Compound E by its m.p. (218–220°, not depressed on admixture with authentic Compound E), the observed absorption maxima in sulfuric acid solution (280–285 mμ, 340 mμ and 410–415 mμ, corresponding to the published maxima for authentic Compound E,[3] the m.p. of the acetate (242–244°[4]), and the analysis of the acetate (Calcd. for $C_{23}H_{30}O_6$: C, 68.65; H, 7.46. Found: C, 68.44; H, 7.20).

A detailed study of this enzymatic reduction including efforts to effect its reversal will be published at a later date

DEPARTMENT OF EXPTL. MEDICINE
JEFFERSON MEDICAL COLLEGE
OF PHILADELPHIA
PHILADELPHIA, PA. JOHN J. SCHNEIDER

RECEIVED MARCH 24, 1953

(1) E. F. Rogers, W. J. Leanza, J. P. Conbere and K. Pfister 3rd, THIS JOURNAL, **74**, 2947 (1952).

(2) The author wishes to thank Dr. Karl Pfister and Dr. E. F. Rogers, Merck and Company, Inc., for the Compound E-21-aldehyde hydrate used in this experiment.

(3) A. Zaffaroni, *ibid.*, **72**, 3828 (1950).

(4) L. H. Sarett, *ibid.*, **70**, 1454 (1948).

ACHROMYCIN.[1] THE STRUCTURE OF THE ANTIBIOTIC PUROMYCIN.[2] I.

Sir:

The new antibiotic, Puromycin, isolated from the mold *Streptomyces alboniger*, has been found to be active against certain bacteria and Trypanosomes.[3]

Puromycin, I, m.p. 175.5–177°, $[\alpha]^{25}D$ − 11° (ethanol), *Anal.* Calcd. for $C_{22}H_{29}N_7O_5$: C, 56.04; H, 6.20; N, 20.79. Found: C, 56.12; H, 6.48; N, 21.12, is a diacidic base and readily forms a dihydrochloride or a monosulfate. Titration and molecular weight data are in agreement with the above empirical formula. Group analyses show the presence of one amino group (Van Slyke), one methoxyl group, two N-methyl groups and five active hydrogens. A carbonyl group is indicated by a band at 6.05 μ in the infrared spectrum. This band may be assigned to a carboxamide grouping. The compound absorbs ultraviolet light with maxima in 0.1 *N* sodium hydroxide at 275 mμ (*E* 20,300) and in 0.1 *N* hydrochloric acid at 267.5 mμ (*E* 19,500). On acid hydrolysis the ultraviolet absorption maxima are shifted 5 to 10 millimicrons to the longer wave length. Concomitantly, the biological activity of the compound is destroyed.

On alcoholysis with ethanolic hydrogen chloride I is cleaved into three fragments. One of these, II, is an amphoteric compound that precipitates as a dihydrochloride, m.p. 225–227° (dec.), from the cleavage mixture. The free base melts at 257–258°, *Anal.* Calcd. for $C_7H_9N_5$: C, 51.52; H, 5.56; N, 42.92; N-methyl, 9.21; mol. wt., 163.2. Found: C, 51.56; H, 5.76; N, 43.05; N-methyl, 14.65; mol. wt. (Rast), 169. The analytical data, the ultraviolet and infrared spectra and the amphoteric nature of II suggest a dimethylamino purine. Compound II was identified as 6-dimethylaminopurine by comparison with an authentic sample.[4]

The second fragment, compound III, was identified as O-methyl-L-tyrosine by analysis and by comparison of its melting point, rotation and spectra with those of an authentic sample. The compound was further characterized by representative derivatives.[5]

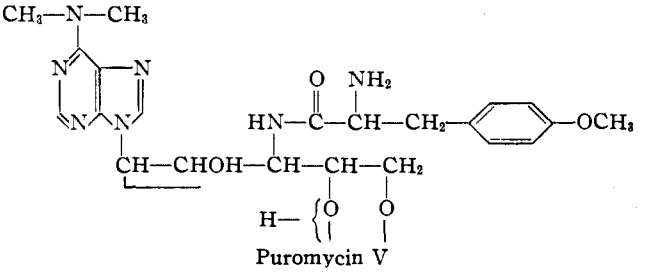
Compound IV, the third fragment, when isolated as its hydrochloride melts at 158–158.5° (dec.), $[\alpha]^{25}D$ −24.6° (water), *Anal.* Calcd. for $C_5H_{11}NO_4 \cdot HCl$: C, 32.35; H, 6.52; N, 7.55; Cl, 19.10; mol. wt., 185.6. Found: C, 32.57; H, 6.72; N, 7.62; amino nitrogen (Van Slyke), 7.70; Cl, 19.45; neut. equiv., 190.7. The characterizing reactions of IV show a positive Fehling test, a positive Brady's reaction, a negative ninhydrin test[6] and the formation of furfural on deamination and subsequent treatment with phosphoric acid. While IV consumes 3.8 moles of periodic acid in three hours, its N-acetyl derivative reacts with 2.0 moles in the same period. The absence of a carbonyl band in the infrared absorption spectrum of IV (in nujol) and the above chemical and analytical data permit the postulation of IV as a hemiacetal form of a 3- or 4-aminopentose.

The formation of a triacetate of I and its subsequent partial deacetylation with alcoholic ammonia to N-acetylpuromycin permits the postulation of two free alcoholic groups in I. The free amino group in I is placed in the O-methyltyrosine moiety by the failure of I to consume periodic acid. This failure to consume periodic acid by I also eliminates a 4-aminopentose structure for compound IV.

The 3-aminopentose was identified as D-3-aminoribose by comparison of its infrared spectrum, melting point and rotation with a synthetic sample.[7]

A negative Brady's test until after hydrolysis indicates a glycosidic linkage in Puromycin. A comparison of the ultraviolet absorption spectra of I with those of 7- and 9-ethyl-6-dimethylaminopurines[8] establishes this linkage to be at the 9 position of the purine.

Partial structure V is proposed for Puromycin, I.



Puromycin V

Structural features of I to be considered in a future communication are: (1) the α- or β-linkage of the glycoside and (2) the furanosidic or pyranosidic nature of the sugar portion.

COY W. WALLER
LEDERLE LABORATORIES DIVISION PETER W. FRYTH
AMERICAN CYANAMID COMPANY BRIAN L. HUTCHINGS
PEARL RIVER, NEW YORK JAMES H. WILLIAMS

RECEIVED MARCH 26, 1953

(1) American Cyanamid Company Trademark for Puromycin.

(2) Puromycin is the generic name for Achromycin.

(3) J. N. Porter, R. I. Hewitt, C. W. Hesseltine, G. Krupka, J. A. Lowery, W. S. Wallace, N. Bohonos and J. H. Williams, *Antibiotics and Chemotherapy*, **2**, 409 (1952).

(4) B. R. Baker, R. E. Schaub, J. P. Joseph and J. H. Williams, to be published.

(5) L. Behr and H. T. Clark, THIS JOURNAL, **54**, 1630 (1932).

(6) 2-Amino sugars give positive ninhydrin test.

(7) B. R. Baker, *et al.*, to be published.

(8) The ultraviolet absorption spectra data for these purines are: 6-dimethylamino-9-ethylpurine $\gamma^{0.1N\ NaOH}_{max}$ 277.5 (*E* 18,300); $\gamma^{.01,N\ HCl}_{max}$ 270 (*E* 17,500); and 6-dimethylamino-7-ethylpurine $\gamma^{0.1N\ NaCl}_{max}$ 295 (*E* 19,400), $\gamma^{0.1N\ HCl}_{max}$ 290 (*E* 23,300), see reference 4.