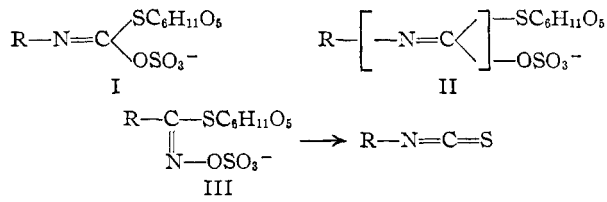
captide containing the elements of sulfate and allyl isothiocyanate, and subsequently on the alkaline hydrolysis of sinigrin to β-D-1-thioglucopyranose.[2] However, I does not accord with the known facts[1,3,4] that sinigrin and analogs yield on direct chemical degradation, not the amines (RNH_2) expected from I, but nitriles (RCN) and carboxylic acids containing the same number of carbon atoms as the enzymatically formed isothiocyanates (RNCS).



I II III

The preceding evidence did establish the general formula II. We now show that version III is the proper expression for the mustard oil glucosides[5]: namely, the decompositions of III or the corresponding silver mercaptide to the nitrile parallel known[6] fissions of thiohydroxamic acids; the formation of the isothiocyanate concurrently with enzymatic removal of the glucosyl group, or nucleophilic abstraction of silver from the mercaptide,[1,3] though novel as an enzyme-initiated process, is an analog of the Lossen rearrangement.[7]

We have proved structure III for sinigrin (R = $H_2C{=}CHCH_2$) and for the glucosinalbate ion (R = p-$HOC_6H_4CH_2$), which is obtained as sinalbin (sinapine glucosinalbate, isolated in 1831) from yellow mustard and furnishes p-hydroxybenzyl isothiocyanate on enzymatic hydrolysis. The glucosinalbate ion was studied as the anhydrous tetramethylammonium salt, m.p. 191–192° (dec.), $[\alpha]^{35}D$ −19° (in water). With Raney nickel in water at room temperature, sinigrin furnished n-butylamine, isolated in 47% yield as the p-nitrobenzamide, and the glucosinalbate furnished tyramine, isolated in 37% yield as the hydrochloride. On acid hydrolysis, sinigrin gave vinylacetic acid, and the glucosinalbate, p-hydroxyphenylacetic acid. Conclusively, acid hydrolyses of sinigrin, sinalbin or the glucosinalbate also afforded *hydroxylamine* in 50–90% yields.[8] Hydrochloric acid at room temperature was effective, but we have mostly used a modification of Yamada's assay[9] for bound hydroxylamine, cleavage in 3 M sulfuric acid containing 2,4-dinitrophenylhydrazine for 2 hours at 95–100°. The hydroxylamine liberated was identified by paper chromatography[10] ($R^{30°F.}$ 0.46–0.51 in 7:3 methanol–6 N hydrochloric acid, detected by three specific reagents), determined quantitatively by the Csaky–Yamada method[9] or as ferric benzhydroxamate,[11] and isolated as fluorenone oxime, m.p. 189–191°, in 13–47% yields from sinigrin and the glucosinalbate.[12]

We conclude: that hydroxylamine derivatives can be accumulated in higher plants[13]; that the skeletal resemblance of amino acids and mustard oil glucosides (*cf.* tyrosine and glucosinalbate) is clarified[14]; that mustard oils are formed in nature by an enzyme-actuated Lossen rearrangement.[14]

We thank Mr. R. W. King, R. T. French Company, for gifts of mustard flour, the National Science Foundation for a predoctoral fellowship (A. J. L.), and The Robert A. Welch Foundation for financial support.

(2) W. Schneider, H. Fischer and W. Specht, *Ber.*, **63**, 2787 (1930). The complete formulation of sinigrin as a β-glucopyranoside is confirmed by the rotation and our desulfurization of tetraacetylsinigrin to tetraacetyl-1,5-anhydro-D-glucitol.

(3) H. Will and W. Koerner, *Ann.*, **125**, 257 (1863); J. Gadamer, *Arch. Pharm.*, **237**, 111, 507 (1899); H. Schmid and P. Karrer, *Helv. Chim. Acta*, **31**, 1017, 1087 (1948).

(4) H. Will and A. Laubenheimer, *Ann.*, **199**, 150 (1879); O. E. Schultz and R. Gmelin, *Arch. Pharm.*, **287**, 342 (1954).

(5) We reject the third possibility, attachment of the thioglucosyl group to the nitrogen atom, because a substance so constituted could afford the isothiocyanate only by an obscure *twofold* shift and would give ammonia on acid hydrolysis, cleaving like known sulfenamides (N. Kharasch, S. J. Potempa and H. L. Wehrmeister, *Chem. Revs.*, **39**, 269 (1946)).

(6) L. Cambi, *Atti reale accad. Lincei, Rend. classe sci. fis., mat. e nat.*, [5] **18**, I, 687 (1909); L. Voltmer, *Ber.*, **24**, 378 (1891).

(7) *Cf.* R. D. Bright and C. R. Hauser, THIS JOURNAL, **61**, 618 (1939). The sulfate residue in III is assigned the configuration *anti* to R, the migrating group.

(8) *Cf.* hydrolyses of ketoxime-O-sulfonic acids: F. Sommer, O. F. Schulz and M. Nassau, *Z. anorg. u. allgem. Chem.*, **147**, 142 (1925); P. A. S. Smith, THIS JOURNAL, **70**, 323 (1948); D. E. Pearson and F. Ball, *J. Org. Chem.*, **14**, 118 (1949).

(9) T. Yamada, *Acta Chem. Scand.*, **9**, 349 (1955).

(10) J. M. Bremner, *Analyst*, **79**, 198 (1954).

(11) G. W. Pucher and H. A. Day, THIS JOURNAL, **48**, 672 (1926).

(12) Progoitrin (M. A. Greer, *ibid.*, **78**, 1260 (1956)), kindly supplied by Dr. Greer, afforded hydroxylamine in 67% assayed yield.

(13) The sinigrin in black mustard is equivalent to a hydroxylamine content of the seed up to *ca.* 0.4%.

(14) Isoleucine and natural (+)2-butyl isothiocyanate, which apparently belong to the same stereochemical series, and the corresponding glucoside could presumably be correlated through (+)2-methylbutyric acid. In the enzymatic formation of this isothiocyanate the migrating carbon atom is asymmetric, and should retain configuration!

DEPARTMENT OF CHEMISTRY
W. M. RICE INSTITUTE MARTIN G. ETTLINGER
HOUSTON 1, TEXAS ALLAN J. LUNDEEN

RECEIVED JULY 5, 1956

THE SYNTHESIS OF ANALOGS OF THE AMINONUCLEOSIDE FROM PUROMYCIN: VARIANTS AT THE 6-POSITION OF THE PURINE MOIETY

Sir:

Since 9-(3-amino-3-deoxy-β-D-ribofuranosyl)-6-dimethylaminopurine (I, R_1 = $N[CH_3]_2$),[1] the aminonucleoside from puromycin[2,3,4] has trypanocidal[5] and tumor-inhibiting properties,[6] analogs were synthesized in which substituents in the 6-position of the purine moiety were varied in order to determine the relation of structure to activity. The synthesis of the 6-amino analog, 3′-amino-3′-deoxyadenosine, from 6-benzamidopurine has been described by Baker, *et al.*[7]

(1) B. R. Baker, J. P. Joseph and J. H. Williams, THIS JOURNAL, **76**, 2838 (1954).

(2) Stylomycin is the registered trade-mark of the American Cyanamid Company for the antibiotic puromycin.

(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) C. W. Waller, P. W. Fryth, B. L. Hutchings and J. H. Williams, THIS JOURNAL, **75**, 2025 (1953).

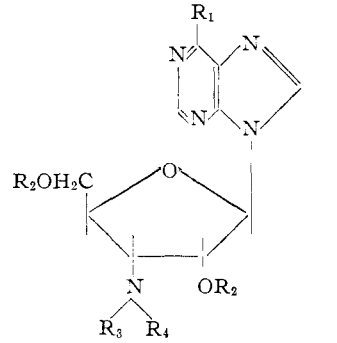
(5) R. I. Hewitt, A. R. Gumble, W. S. Wallace and J. H. Williams, *Antibiotics and Chemotherapy*, **4**, 1222 (1954).

(6) P. L. Bennett, S. L. Halliday, J. J. Oleson and J. H. Williams, "Antibiotics Annual 1954–1955," Medical Encyclopedia, Inc., New York, N. Y., 1954, pp. 766–769.

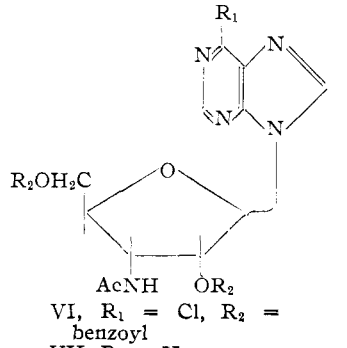
(7) B. R. Baker, R. E. Schaub and H. M. Kissman, THIS JOURNAL, **77**, 5911 (1955).

A mixture of chloromercuri-6-chloropurine (II)[8] and bis-(6-chloropurinyl)mercury (III), from reaction of 6-chloropurine with mercuric chloride and sodium hydroxide, was condensed with 2,5-di-*O*-benzoyl - 3 - deoxy - 3 - phthalimido - β - D - ribofuranosyl chloride[9] in boiling xylene to give 64% of **9-(2,5-di-*O*-benzoyl-3-deoxy-3-phthalimido-β-D-ribofuranosyl)-6-chloropurine** (IV) ($^1/_4CH_3COOC_2H_5$, sinters 70–72° to an opaque glass, m.p. 100–105° to a clear glass, $[\alpha]^{24}D$ − 62.0° [c = 2 ($CHCl_3$)], found: C, 61.4; H, 3.96; Cl, 5.38; N, 10.8; OC_2H_5, 1.67).

Condensation by means of titanium tetrachloride of a mixture of mercury derivatives (II) and (III) with 3-acetamido-1-*O*-acetyl-2,5-di-*O*-benzoyl-3-deoxy-D-ribofuranose[10] in boiling ethylene dichloride gave 85% of an anomeric mixture of **9-(3-acetamido-2,5-di-*O*-benzoyl-3-deoxy-α- and β-D-ribofuranosyl)-6-chloropurines** (VI) as a crude glass ($[\alpha]^{24.5}D$ + 39.2° [c = 1 ($CHCl_3$)], found: C, 57.8; H, 4.52; Cl, 6.59; N, 11.4).



I, $R_2 = R_3 = R_4 = H$
IV, R_1 = Cl, R_2 = benzoyl, R_3—R_4 = phthaloyl
V, $R_1 = N[alkyl]_2$, $R_2 = R_3 = H$, $R_4 = COC_6H_4$-*o*-$CON[alkyl]_2$



VI, R_1 = Cl, R_2 = benzoyl
VII, $R_2 = H$

Chloronucleosides (IV) and (VI), reacting with methanolic *primary* alkyl amines under reflux or at 100° in sealed tubes, simultaneously underwent displacement of chloride by amine, removal of *O*-benzoates as methyl benzoate and removal of the phthaloyl blocking group of (IV) as N,N′-disubstituted phthalamides to produce unblocked aminonucleosides (I, R_1 = NH = alkyl), and to produce 3′-acetamidonucleosides (VII, R_1 = NH = alkyl) from (VI). Chloronucleosides (IV) and (VI), reacting with methanolic *secondary* alkyl amines, simultaneously underwent displacement of chloride by amine, removal of *O*-benzoates as methyl benzoate, opening of the phthalimide (IV) to produce 3′-deoxy-3′-(*o*-N,N-disubstituted carbamoyl)-benzamidonucleosides (V), and to produce 3′-acetamidonucleosides (VII, $R_1 = N[alkyl]_2$) from (VI). Reaction of the benzamidonucleosides (V) with methanolic *primary* alkyl amines produced unblocked aminonucleosides (I, $R_1 = N[alkyl]_2$). Reaction of chloronucleosides with nucleophilic reagents other than ammonia[11] has not been previously described.

Structural proof of the 6-chloro-3′-phthalimidonucleoside (IV) was obtained by treating with methanolic dimethylamine and then with methanolic methylamine to give 68% of (I, $R_1 = N[CH_3]_2$) (m.p. 214–216°, $[\alpha]^{25}D$ −23.9° [c = 2 (H_2O)]), identical with an authentic sample of the aminonucleoside[1] from puromycin. That the 3′-acetamido-6-chloronucleoside (VI) is an anomeric mixture was shown by reaction with methanolic methylamine to produce 15% of the **α-anomer**[12] of the **3′-acetamido-6-methylamino analog** (VII α, $R_1 = NHCH_3$) ($^1/_4$ hydrate, m.p. 257–258° dec., $[\alpha]^{28}D$ +114° [c = 1 (H_2O)], found: C, 47.8; H, 6.03; N, 26.1) and 26% of the **β-anomer**[12] of the **3′-acetamido-6-methylamino analog** (VII β, $R_1 = NHCH_3$) ($^1/_4$ hydrate, m.p. 229–230° dec., $[\alpha]^{28}D$ − 2.0° [c = 1 (H_2O)], found: C, 48.0; H, 6.05; N, 26.0).

Reaction of (IV) with methanolic methylamine gave 84% of the **β-anomer** of the **6-methylamino analog** (I, $R_1 = NHCH_3$); barium hydroxide hydrolysis of an anomeric mixture of the **3′-acetamido-6-methylamino analog** (VII α + β, $R_1 = NHCH_3$) gave 4% of the **β-anomer** of the **6-methylamino analog** (I, $R_1 = NHCH_3$) (m.p. 230–231°, $[\alpha]^{25}D$ − 26.9° [c = 1 (H_2O)], found: C, 47.0; H, 6.00; N, 29.7).

Reaction of (VI) with methanolic dimethylamine afforded 52% of (VII β, $R_1 = N[CH_3]_2$) (m.p. 186–189°, $[\alpha]^{25}D$ − 8.1° [c = 2 (pyridine)]), identical with an authentic sample of the N-acetyl derivative[10] of the aminonucleoside from puromycin.

Reaction of (IV) with methanolic diethylamine followed by methanolic butylamine gave 69% of the **6-diethylamino analog** (I, $R_1 = N[C_2H_5]_2$) (m.p. 181.5–183°, $[\alpha]^{25}D$ − 48.6° [c = 1 (EtOH)], found: C, 52.2; H, 7.07; N, 26.3). Acetamidochloronucleoside (VI), reacting with methanolic diethylamine, gave 47% of the **3′-acetamido-6-diethylamino analog** (VII β, $R_1 = N[C_2H_5]_2$) (m.p. 214.5–215°), $[\alpha]^{24.5}D$ − 26.0° [c = 0.7 (EtOH)], found: C, 52.9; H, 6.99; N, 22.8). Barium hydroxide hydrolysis of the latter gave 62% of the **6-diethylamino analog** (I, $R_1 = N[C_2H_5]_2$), m.p. 181–183°.

Trypanocidal Activity.[14]—Of a large number of 6-substituted analogs synthesized the following are listed together with their activities against *Trypano-*

(8) G. B. Brown and V. S. Weliky, *J. Biol. Chem.*, **204**, 1019 (1953).

(9) B. R. Baker, J. P. Joseph and R. E. Schaub, THIS JOURNAL, **77**, 5905 (1955).

(10) B. R. Baker, R. E. Schaub, J. P. Joseph and J. H. Williams, *ibid.*, **76**, 4044 (1954).

(11) *Cf.* E. Fischer and B. Helferich, *Ber.*, **47**, 210 (1914); J. Davoll and B. A. Lowy, THIS JOURNAL, **73**, 1650 (1951); ref. (8).

(12) Based on Hudson's rules for sugars[13] the most dextrorotatory anomer of the D-series is assigned the α-configuration; for extension to nucleosides *cf.* Baker, *et al.*[7]

(13) C. S. Hudson, THIS JOURNAL, **60**, 1537 (1938).

(14) Private communication from Dr. R. I. Hewitt of these Laboratories.

soma equiperdum in mice as compared with the aminonucleoside from puromycin rated 1: **isobutylamino,** 2; **(methyl)-propylamino,** 4; **diethylamino,** 4–8; **(ethyl)-propylamino,** 2; **dipropylamino,** 4–8; **diallylamino,** 1; **(butyl)-ethylamino,** 1; **cyclohexylamino,** < 0.5; **1-piperidyl,** 0.25; **benzylamino,** < 0.25; **furfurylamino,** 0.5.

ORGANIC CHEMICAL RESEARCH SECTION
RESEARCH DIVISION
AMERICAN CYANAMID COMPANY
LEDERLE LABORATORIES
PEARL RIVER, NEW YORK

LEON GOLDMAN
JOSEPH W. MARSICO
ROBERT B. ANGIER

RECEIVED JULY 3, 1956

A POSSIBLE ENZYMATIC ROLE OF ERGOTHIONEINE[1]

Sir:

Previous reports from this laboratory have shown the existence of essentially two types of enzymes which split DPN at the nicotinamide–ribose linkage. One type is nicotinamide sensitive, or inhibited by nicotinamide, such as the DPNases from beef spleen[2] and pig brain,[3,4] the second type is relatively unaffected by the presence of nicotinamide as exemplified by the *Neurospora* DPNase.[5]

We have recently described[6] a nicotinamide-sensitive enzyme from human and bovine erythrocytes which splits nicotinamide riboside (NR). Partial purification of this enzyme, from hemolysates, has been effected by fractional ammonium sulfate precipitation. Purification of the nicotinamide ribosidase resulted in retention of the splitting activity of NR with complete loss of the nicotinamide sensitivity. However, the nicotinamide sensitivity could be restored to the partially purified enzyme by reconstitution with a 5% trichloroacetic acid extract of the intact washed erythrocytes, or by the supernatant fraction resulting from the ammonium sulfate precipitation of the enzyme.

The same factor from the erythrocytes could also impart nicotinamide sensitivity to the *Neurospora* DPNase. This DPNase does not promote an exchange reaction of C^{14}-nicotinamide for the nicotinamide moiety of DPN. However the addition of the factor can endow the *Neurospora* enzyme with exchange properties. Evidence has also been obtained that the nicotinamide ribosidase from erythrocytes will promote an exchange reaction only in the presence of the factor.

Preliminary chemical studies revealed that the factor contained a sulfhydryl grouping which was inactivated by bromine oxidation, or by metal ions. Enzymatic activity of the oxidized product was not restored by the addition of reducing agents. Inactivation by metal ions, however, did respond to reactivation by either glutathione, cysteine or ascorbic acid.

That the factor showed sulfhydryl properties led us to try a number of known sulfur compounds for activity. The only compound which showed activity in promoting nicotinamide sensitivity to the nicotinamide ribosidase and the *Neurospora* DPNase was ergothioneine (the betaine of 2-thiolhistidine). The concentrations of authentic ergothioneine (1×10^{-5} *M*) utilized routinely for demonstrating nicotinamide sensitivity, associated with the *Neurospora* DPNase and erythrocyte nicotinamide ribosidase, were of the same order of magnitude as that contained in the trichloroacetic acid extract. Glutathione, cysteine, coenzyme A or 2-thiolhistidine were without effect.

Authentic ergothioneine (Mann Research Laboratories, Inc.) and the factor from washed erythrocytes behaved similarly when chromatographed on an anionotropic aluminum oxide (M. Woelm Eschwege, Alupharm Chemical) column eluted with an ethanol–formic acid mixture according to the directions of Melville, Horner and Lubschez.[7] The resolution of the biologically active material, from the trichloroacetic acid extract as seen in Fig. 1, was coincident with material reacting positively to the Hunter test,[8] as modified by Melville and Lubschez,[9] for ergothioneine and 2-thiolhistidine.

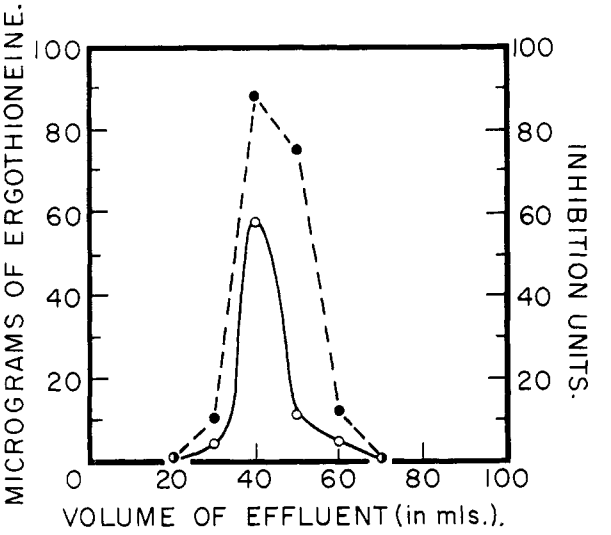


Fig. 1.—Partition chromatography of the trichloroacetic acid extract of human erythrocytes: inhibition units represent the per cent. inhibition of nicotinamide ribosidase in the presence of 10^{-2} *M* nicotinamide and a 0.1-ml. sample from each 5-ml. fraction collected. All enzymatic reaction mixtures contained 3 units of nicotinamide ribosidase (1 unit is that quantity of enzyme which will split 0.1 micromole of NR in 6 minutes), 10^{-5} *M* glutathione, 0.1 *M* phosphate buffer (*p*H 7.4) and water to a final volume of 0.7 ml. A preincubation period of 5 minutes was followed by the addition of 1.4 micromoles of NR. The dashed line represents the inhibition units whereas the solid line depicts the ergothioneine concentration.

(1) Research contribution of the McCollum-Pratt Institute, aided by grants from the National Cancer Institute of the National Institutes of Health (Grant No. C-2374C), and from the American Cancer Society as recommended by the Committee on Growth of the National Research Council.

(2) L. J. Zatman, N. O. Kaplan and S. P. Colowick, *J. Biol. Chem.*, **200**, 197 (1953).

(3) L. J. Zatman, N. O. Kaplan, S. P. Colowick and M. M. Ciotti, *ibid.*, **209**, 453 (1954).

(4) N. O. Kaplan and M. M. Ciotti, *ibid.*, in press.

(5) N. O. Kaplan, S. P. Colowick and A. Nason, *ibid.*, **191**, 473 (1951).

(6) L. Grossman, *Fed. Proc.*, **15**, 266 (1956).

(7) D. B. Melville, W. H. Horner and R. Lubschez, *J. Biol. Chem.*, **206**, 221 (1954).

(8) G. Hunter, *Canad. J. Res.*, Sec. E, **27**, 230 (1949).

(9) D. B. Melville and R. Lubschez, *J. Biol. Chem.*, **200**, 275 (1953).