lized from a mixture of benzene and ligroin, giving 465 mg. of colorless crystals, m.p. 159–161°, undepressed by admixture of starting material.

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N-Substituted Colchiceinamides

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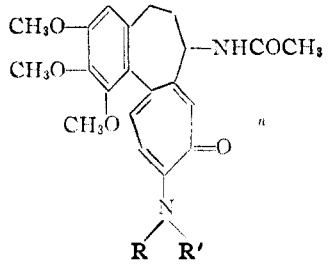
Since certain N-substituted colchiceinamides[2] have been reported to inhibit cell mitosis[3] and growth[4] in certain tumors, we have prepared a series of these derivatives as listed in the table, for screening against Sarcoma 37 in mice. Six of the fourteen compounds are new; of the other eight, analyses are not given in the literature for six, the melting points for two are not listed, and the melting points reported for three others are widely different from those reported here. It was therefore thought desirable to bring all the characterizing data together.

With the exception of the β-chloroethyl derivative, all the compounds were prepared generally according to the method of Zeisel[5] by heating colchicine with a 10% alcoholic solution of the appropriate amine in 50% excess in a sealed tube at 120° (100° for colchiceinamide itself) for varying lengths of time depending on the amine. The reaction mixtures were evaporated to dryness and the products crystallized from suitable solvents.

N-SUBSTITUTED COLCHICEINAMIDES



Substituent	Reaction time, hr.	Appearance, crystallizing solvent	M.p., °C. cor.	Yield, % Crude	Yield, % Pure	Empirical Formula	Analyses,[k,l] % Methoxyl Calcd.	Methoxyl Found	Nitrogen Calcd.	Nitrogen Found
None[a,b]	4	Prisms, alc.	261–262	82	63	$C_{21}H_{24}N_2O_5$	24.2	24.0	7.3	7.0
Methyl[b,c]	20	Prisms, EtOAc	230–232 (softens 185)	85	66	$C_{22}H_{26}N_2O_5$	23.4	23.1	7.0	6.8
Ethyl[b,d]	20	Needles, EtOAc	200–210 (softens 94)	87	82	$C_{23}H_{28}N_2O_5$	22.6	22.2	6.8	6.8
n-Propyl[b,e]	18	Prisms, alc.	162–165	..	61	$C_{24}H_{30}N_2O_5$	21.8	22.5	6.6	6.3
n-Butyl[b,f]	18	Prisms, alc.	192–193	87	75	$C_{25}H_{32}N_2O_5$	21.1	20.7	6.4	6.3
n-Amyl	18	Prisms, alc.	189–194	..	98	$C_{26}H_{34}N_2O_5$	20.5	20.5	6.2	6.0
n-Hexyl	18	Needles, benz.	164–166 (softens 157)	90	..	$C_{27}H_{36}N_2O_5$	19.9	20.5	6.0	6.0
n-Heptyl	18	Amorphous[j]	131 (softens 94)	83	..	$C_{28}H_{38}N_2O_5$	19.3	19.4	5.8	5.8
n-Octyl	18	Amorphous[j]	121 (softens 85)	77	..	$C_{29}H_{40}N_2O_5$	18.7	18.6	5.6	5.8
β-Hydroxyethyl[g]	20	Prisms, EtOAc	225–226 (softens 185)	86	51	$C_{23}H_{28}N_2O_6$	21.7	21.2	6.5	6.5
β-Chloroethyl	..	Amorphous	...	94	..	$C_{23}H_{27}ClN_2O_5 \cdot 2H_2O$	18.3	18.8	[m]	
Dimethyl[h]	20	Amorphous	203–205 (foams 145)	89	68	$C_{23}H_{28}N_2O_5$	22.6	22.6	6.8	6.6
Diethyl[i]	26	Needles, EtOAc & pet. ether	209–211	86	75	$C_{25}H_{32}N_2O_5$	21.1	21.4	6.4	6.1
Bis-(β-hydroxy)-ethyl	26	Amorphous	...	47	34	$C_{25}H_{32}N_2O_7$	19.7	19.5	5.9	6.2

[a] Reference 1; dimorphic crystals from ethanol analyzing for 0.5 mole ethanol of crystallization. No m.p. given. [b] H. Lettré, *Naturwissenschaften*, **33**, 75 (1946). No m.p. or anal. given. [c] May and Baker, Ltd., *et al.*, British Patent 577,606 (1946); prisms from ethanol–ether, m.p. 173–174°. No anal. given. [d] See ref. [c]; prismatic needles from ether, m.p. 160–162°. No anal. given. [e] See ref. [c]; prisms from ether, m.p. 164°. No anal. given. [f] See ref. [c]; prisms from benzene–ether, m.p. 196°. No anal. given. [g] See ref. [c]; amorphous. No m.p. or anal. given. [h] See ref. [c]; micro crystals, m.p. 204–206°. No anal. given. More recently this compound has been described by H. Rapoport and A. R. Williams, THIS JOURNAL, **73**, 1896 (1951), as having m.p. 174–176°, and its constitution was confirmed by analysis. [i] See ref. [c]; prisms from alcohol–ether, m.p. 207°. No anal. given. [j] Further treatment by chromatography in chloroform solution over activated alumina did not yield a crystalline product. [k] By the Microanalytical Laboratory, National Institutes of Health, in charge of Dr. W. C. Alford. [l] Difficulty was experienced in burning most of these compounds in order to obtain C and H percentages. Since colchicine itself, which would be the expected impurity, has the calculated values OCH_3, 31.1 and N, 3.5, methoxyl and nitrogen analyses represent valid criteria of purity. [m] Chlorine analysis: calcd., 7.5; found 7.4. [n] This formula is based on what is regarded as the most likely structure for colchicine. As an alternative, the substituents in the "C" ring may be reversed, with appropriate shifts of the double bonds.

The β-chloroethyl derivative was prepared from the β-hydroxyethyl derivative by the action of thionyl chloride. While nearly all the compounds were obtained crystalline, it was found that these crystals gave erratic analytical results, probably due to retention of small amounts of solvent,

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(2) Colchiceinamide has been more usually called colchicamide and colchicinamide. It seemed to us more logical to base the name on the "acid" colchiceine rather than on colchicic acid, a name which has been given to two compounds, or on the "ester" colchicine.

(3) H. Lettré, *Die Chemie*, **55**, 265 (1942); H. Lettré and H. Fernholz, *Z. physiol. Chem.*, **278**, 175 (1943).

(4) H. Lettré, *Z. Krebsforsch.*, **57**, 1 (1950).

(5) S. Zeisel, *Monatsh.*, **9**, 1 (1888).

whereas drying under vacuum at temperatures around 100° produced decomposition. Consequently, for analysis, the crystalline products were dissolved in chloroform and precipitated with light petroleum ether or *n*-hexane; this procedure yielded products apparently free of retained solvents. All the compounds were yellow in color, and all gave water-soluble hydrochlorides with the exception of the derivatives butyl through octyl.

Some activity against Sarcoma 37 in mice was exhibited by all of the compounds.[6]

Experimental

The colchicine used had a m.p. of 158–159° cor. and was purified[7] by us from a commercial product employing chromatography over activated alumina. The amines were the best grades of Eastman Kodak Co., Sharples Chemicals Inc., and Fischer Scientific Co. with the exception of hexylamine (Eastman Kodak Co., practical), and of diethanolamine (Carbon and Carbide Chemicals Corp., practical); methylamine, ethylamine and dimethylamine were used as the concentrated aqueous solutions.

N-(β-Chloroethyl)-colchiceinamide.—To a solution of 0.43 g. (0.001 mole) of N-(β-hydroxyethyl)-colchiceinamide in 200 cc. of dry, thiophene-free benzene, cooled to 20°, was added dropwise with vigorous stirring 0.1 cc. of purified[8] thionyl chloride. After standing overnight in the refrigerator, the supernatant liquid was decanted off, the residue washed with benzene by decantation, and the crude yellow product dried in a vacuum desiccator; yield 0.45 g. (94%). The compound was purified by dissolving in chloroform, precipitating with twenty volumes of absolute ether, washing the solid with ether and drying in vacuum at 55°. The amorphous substance is hygroscopic.

Attempts to prepare the N,N-bis-(β-chloroethyl) derivative from N,N-bis-(β-hydroxyethyl)-colchiceinamide in a similar fashion yielded an extremely hygroscopic gummy product which always contained less than the theoretical amount of chlorine and could not be satisfactorily purified.

(6) To be reported in the *Journal of the National Cancer Institute.*
(7) J. N. Ashley and J. O. Harris, *J. Chem. Soc.*, 677 (1944).
(8) L. F. Fieser, "Experiments in Organic Chemistry," 2nd edition, D. C. Heath and Co., Boston, Mass., 1941, p. 381.

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Amino Acid Composition of Crystalline Inorganic Pyrophosphatase Isolated from Bakers Yeast

BY WERNER HAUSMANN

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Inorganic pyrophosphatase has recently been isolated by Dr. M. Kunitz in crystalline form.[1,2] It appeared of interest to investigate the amino acid composition of the new enzyme qualitatively and quantitatively by hydrolysis and chromatography of the hydrolysate.

Qualitative Determination.—A good qualitative picture of the amino acid spectrum of the protein was obtained by paper chromatography.

Five mg. of air-dried crystalline pyrophosphatase was hydrolyzed in 1 ml. of 6 *M* HCl for 24 hours at 110°, in a sealed, evacuated Pyrex glass tube. The hydrolyzate was then evaporated to dryness at 50° and 9 mm. pressure, redissolved in 1 ml. of distilled water and evaporated again in order to remove excess HCl. The remaining mixture of amino acid hydrochlorides was dissolved in 0.5 ml. of distilled water to give a concentration corresponding to 10 γ of original protein per μl.

Twenty μl. (200 γ) of this solution was subjected to two dimensional paper chromatography on Whatman No. 1 filter paper, using the ascending technique.[3] The solvent system used first for running along the longer edge of the paper consisted of 150 ml. of redistilled secondary butanol +60 ml. of 3% aqueous ammonia. This was done twice before turning the paper and running it once along the short paper edge in the second solvent system: 150 ml. of distilled secondary butanol +30 ml. of 88% aqueous formic acid +20 ml. of water. After drying, the paper was held in a horizontal position and sprayed with ninhydrin solution. After five minutes, when the paper looked dry, the cystine region was sprayed with Folin reagent.[4] The area of arginine was sprayed with Sakaguchi solution.[5] Control runs proved that these two specific color reactions were positive even after ninhydrin treatment, if applied immediately.

For comparison a synthetic mixture of 20 amino acid hydrochlorides was prepared, and chromatographed in exactly the same manner. The result is illustrated in Fig. 1. Proline and hydroxyproline give yellow spots. For more accurate comparison the hydrolyzate was run together with standard samples of the 16 amino acids indicated. No new spots appeared.

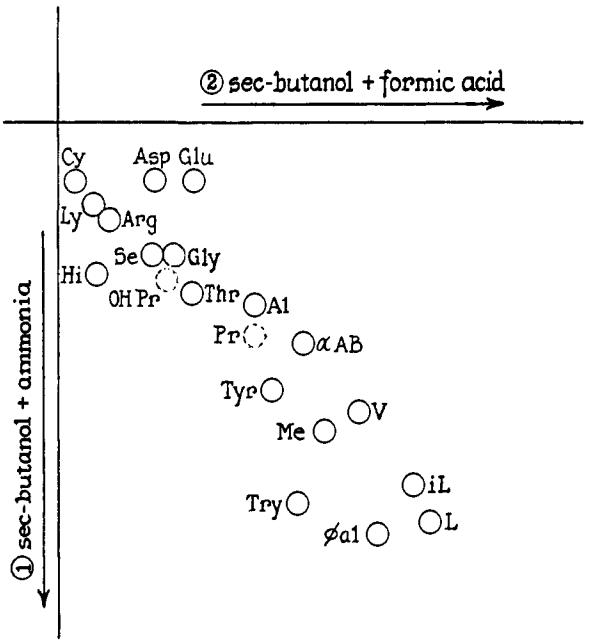

Fig. 1.—Synthetic mixture of amino acid hydrochlorides.

Cystine could not be detected either by ninhydrin or Folin treatment.

Reaction of the intact protein with *p*-dimethylaminobenzaldehyde[6] indicated the presence of tryptophan. This was confirmed by paper chromatography of a $Ba(OH)_2$ hydrolyzate.

Paper chromatography has revealed that crystalline inorganic pyrophosphatase is a protein containing the following 17 amino acids: aspartic acid, glutamic acid, lysine, arginine, histidine, serine, threonine, proline, methionine, tyrosine, tryptophan, glycine, alanine, valine, leucine, isoleucine and phenylalanine. Cystine and hydroxyproline could not be detected.

Quantitative Determination.—The quantitative amino acid composition was determined on the above mentioned hydrolyzate in HCl by chromatography on Dowex 50 columns,[7] and the fractions were analyzed by the colorimetric ninhydrin method.

Figures 2 and 3 represent patterns obtained by the 100-cm. and the 15-cm. columns, respectively. The qualitative

(1) M. Kunitz, THIS JOURNAL, **73**, 1387 (1951).
(2) M. Kunitz, *J. Gen. Physiol.*, **35**, 423 (1952).
(3) R. J. Williams, and H. Kirby, *Science*, **107**, 481 (1948).
(4) O. Folin and J. M. Looney, *J. Biol. Chem.*, **51**, 421 (1922).
(5) E. Jorpes and S. Thoren, *Biochem. J.*, **26**, 1504 (1932).
(6) J. R. Spies and D. C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).
(7) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).