skin prick blood without clotting. The gaseous phase is not disturbed by removal of the stopper when collecting finger prick blood and in any case is more important in maintaining the pH of unused media than that of inoculated cultures, which are rapidly conditioned upon addition of the whole blood.

The inoculated culture may be left up to 48 hours at 4°C before incubating. Storage at room temperature before incubation slows but does not halt cell division so that harvesting should take place 2 to 4 days after inoculating rather than after incubation at 37°C. During the 20 minutes of treatment with citrate, the swollen cells sediment by gravity and the subsequent centrifugation is meant only to pack them slightly in order to ease the removal of hypotonic fluid without disturbance. Prolonging the centrifugation will not increase the yield of cells. Cloudiness in the supernate can be disregarded. If necessary the cells may be left in fixative for days before preparation of slides.

The virtues of this method are, first, the use of a minimum of materials and equipment. Most of the latter is already in standard hospital use and only one tube is used throughout. Second, the method of collection and culture is flexible, being equally suitable for infants, children and adults, as well as for experimental animals, both in and out of hospital, times of inoculation and incubation being adjusted to suit circumstances. Third, the combination of economy and flexibility means that the fundamental information to be gained from population, as opposed to case, studies should now be available without crippling expense, especially if viewed in combination with computer techniques for karyotypic analysis(1).

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Beta-Hydrazinopropionitriles: A New Series of Osteolathyrogenic Agents for the Rat.* (29949)

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In the course of screening a variety of different types of compounds for osteolathyrogenic activity in the weanling rat, we found that β-hydrazinopropionitrile (BHPN), 2-cyanoethylhydrazine, caused lathyritic skeletal changes similar to those produced by β-aminopropionitrile (BAPN). In an attempt to relate specific details of structure to lathyrogenicity, we have also studied the lathyrogenic actions of a few methyl-substituted derivatives of BHPN.

Methods. Milled Rockland Rat Stock Diet served as the basal diet. To this were added varying amounts of the BHPN under test. Male Holtzman rats weighing 55 to 65 g were fed these diets and examined after varying periods for skeletal abnormalities and aortic damage. In addition, hydroxyproline levels were determined in saline-soluble and insoluble collagen fractions of sponge biopsies from adult rats which were fed these substances and pair-fed with normal controls. For the latter determinations, polyvinyl surgical sponges were implanted subcutaneously as previously described(1). In some instances the animals were pre-fed the experimental diets one day before sponge implantation and sacrificed at the indicated intervals; in more recent trials sponges were implanted into normal rats, experimental diets were started 19 days after implantation and the animals were sacrificed 10 days later. Similar results were obtained by both methods. After the sponges were removed, they were frozen solid. They were then homogenized in 2 ml of water containing one drop of octanol-1 by means of a high-speed Virtis "45" homogenizer using 3 micro blades on a micro aerosol-free assembly

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and using a 5 ml transition flask immersed in an ice-water bath. Each sponge tissue suspension, together with two 1-ml rinsings of water, was centrifuged at 2°C and the supernatant solution discarded. The residue was suspended in 3 ml of 0.5 *M* neutral NaCl by means of a Vortex Jr. mixer and extracted overnight at 2°C. Following centrifugation at 2° in a refrigerated centrifuge, the residue was extracted twice more at 2° with 2 ml portions of 0.5 *M* NaCl for 2 hours each time and centrifuged. The combined supernates from these 3 extractions represent *saline-soluble collagen.* The residue from the third saline extraction was suspended in 3 ml of water, autoclaved for 3 hours and centrifuged at room temperature. The residue was then twice resuspended in 2 ml of water, boiled 10 minutes each time, and centrifuged. The combined 3 extracts represent *insoluble collagen.* Both soluble and insoluble fractions were evaporated to dryness at 105°C in an air jet, were hydrolyzed in sealed tubes with 6 *N* HCl at 104°C for 20 hours, and were neutralized with 2.5 *N* NaOH. After filtration and dilution, they were analyzed for hydroxyproline by the method of Neuman and Logan(2). Hydroxyproline content was taken as a measure of collagen.

Materials. Mono-β-aminopropionitrile (BAPN) *fumarate* was a gift from Abbott Laboratories, North Chicago, Ill.

β-Hydrazinopropionitrile (BHPN) (2-cyanoethylhydrazine) was prepared by reacting acrylonitrile with hydrazine hydrate(3) and converting to the hydrochloride. The structure was confirmed by elemental analysis, NE (neutralization equivalent), and NMR (nuclear magnetic resonance) and IR (infrared) spectra.

α-methyl-BHPN was prepared by reacting methacrylonitrile with hydrazine hydrate and converted to the sulfate: m.p. 159°C (decomp); NE 147.1 (Calcd. 148.1); Anal., N, 27.25 (Calcd. 28.36); structure confirmed by NMR spectrum.

β-methyl-BHPN was prepared by reacting 3-butenenitrile or crotononitrile with hydrazine hydrate in the cold and then converted to the hydrochloride: m.p. 56-60°C; NE 137.9 (Calcd. 135.6); Anal., N, 30.89 (Calcd. 30.99); structure confirmed by NMR and IR spectral analyses. If the reaction mixture is heated to 100°, this compound cyclizes to form 3-amino-5-methyl-pyrazoline (structure of the hydrochloride confirmed by NMR and IR spectra). The hydrochloride of this pyrazoline was not lathyrogenic when fed to weanling rats at levels of 0.3 and 0.5% of the diet and is not included in the results below.

N^1-methyl-BHPN (3-(1-methylhydrazino)-propionitrile) ($H_2N \cdot N(CH_3) \cdot CH_2 \cdot CH_2 \cdot CN$) was prepared by reacting acrylonitrile with methylhydrazine and converted to the hydrochloride: m.p. 116-120°; NE 137.1 (Calcd. 135.6); Anal., C, 35.58 (Calcd. 35.42), H, 6.99 (Calcd. 7.43), N, 30.86 (Calcd. 30.99); structure confirmed by NMR and IR spectra. Further confirmation of this structure was obtained from elemental analysis of the hydrazone obtained by reacting the N-methyl-BHPN with *p*-nitrobenzaldehyde and IR spectral analysis of this hydrazone.

BHPN and its methyl derivatives were rigorously purified and stored in the freezer to prevent deterioration. Deteriorated samples showed increased toxicity and could often not be fed at lathyrogenic levels.

Results and discussion. The effects of feeding the compounds in this series are shown in Fig. 1 and in Table I. Judged on the basis of its skeletal effects (formation of exostoses, spinal curvatures, etc.), BHPN is at least as potent an osteolathyrogenic agent as BAPN. BHPN is more toxic than BAPN however, since dietary levels of 0.25% of the hydrochloride were lethal to weanling rats. Even levels of 0.15 and 0.1% of BHPN · HCl caused severe growth depression.

The osteolathyrogenic activity of α-methyl-BHPN sulfate appeared to be about the same as that of BHPN · HCl. It also caused marked growth depression at 0.1 and 0.15% dietary levels and could not be fed to young rats above the 0.15% level.

The β-methyl-BHPN, while less potent than BHPN or α-methyl-BHPN, was also definitely osteolathyrogenic (Fig. 1).† Lathy-

† Our previous report regarding the non-lathyrogenicity of β-methyl-BHPN(4) was in error. We later found that the compound we were then testing was the pyrazoline derivative described above.

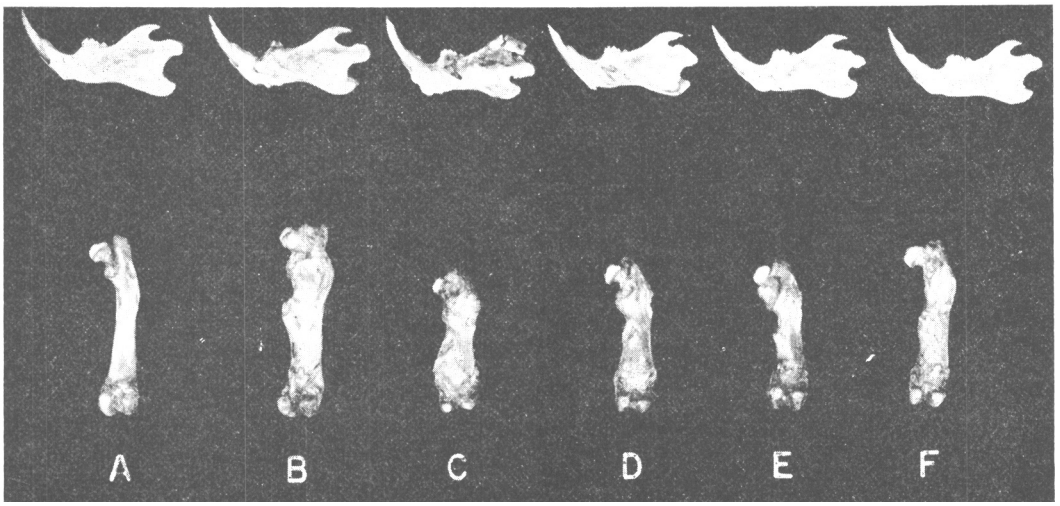

FIG. 1. Effect of BHPN compounds on femurs and mandibles in rats. da = days on experiment. ( ) = initial and final weights of rats. A = normal, 21 da (60-187); B = 0.15% BAPN • fumarate, 31 da (55-177); C = 0.15% BHPN • HCl, 23 da (58-82); D = 0.15% α-methyl-BHPN • ½ H_2SO_4, 23 da (58-123); E = 0.4% β-methyl-BHPN • HCl, 28 da (59-102); F = 0.4% N^1-methyl-BHPN • HCl, 36 da (65-116).

rogenic levels (*e.g.*, 0.4% as the hydrochloride) caused growth depression.

Mild osteolathyrogenicity was also exhibited by N^1-methyl-BHPN (Fig. 1) both when fed as the free base (0.3% level) and as the hydrochloride (0.4%). These levels also caused a growth depression in weanling rats.

Table I shows that all the compounds here reported increased the saline-soluble collagen fractions in sponge biopsy tissue. Increase in salt-soluble collagen is accepted by many as

TABLE I. Effect of Feeding BHPN Derivatives on Collagen Fractions of Sponge Biopsy Tissues from Adult Rats.

Chemical fed, %	Days on diet	μg hydroxyproline/mg wet tissue[a] Saline fraction	Insoluble fraction
.20 BAPN • fumarate[b,c]	14	.3019 (.1017)	2.785 (2.290)
	21	.7243 (.1514)	3.887 (3.434)
Idem[d]	10	.3280 (.1428)	3.906 (4.134)
.15 BHPN • HCl[b,c]	14	.2112 (.1017)	1.886 (2.290)
	21	.4686 (.1514)	3.560 (3.434)
Idem[d]	10	.4446 (.1750)	3.568 (3.184)
.15 α-methyl-BHPN • ½ H_2SO_4[b,c]	12	.1424 (.0842)	1.718 (2.706)
	21	.4674 (.1516)	4.023 (4.174)
.40 β-methyl-BHPN • HCl[b]	12	.1471 (.0660)	2.131 (1.605)
	23	.2315 (.1804)	2.958 (3.659)
.50 N^1-methyl-BHPN • HCl[d]	10	.4113 (.1369)	4.053 (3.107)
.20 BAPN • fumarate + .25 BHPN • HCl[b,c]	16	.7928 (.0858)	1.640 (2.522)
	23	1.339 (.1535)	1.444 (3.149)

[a] All values are averages of 2 sponges; averages for pair-fed normal controls given in parentheses.

[b] Animals pre-fed exp diets 1 day before sponge implantation and sacrificed at indicated intervals.

[c] Sponge tissue extracted with 1.0 *M* NaCl in place of 0.5 *M* NaCl. Both methods gave similar results.

[d] Animals pre-implanted with sponges 19 days, then fed exp diets 10 days.

an index of lathyrogenicity. The administration of BAPN and BHPN together appeared to give rise to saline-soluble collagen levels higher than those produced by either compound alone.

None of this series of BHPN compounds gave rise to ruptured aortae, nor were any gross aneurysms found in any of the sacrificed animals. However, occasional microscopic sections of aortae were found which showed disorganized or fragmented elastic fibers in the media (Hornowski stain). This was true of some animals fed each of the BHPN compounds tested, indicating that each of these substances can cause at least mild aortic damage.

Not tested was the methyl-substituted BHPN having a methyl group on the terminal nitrogen atom of the hydrazino group. This compound would be of particular interest in view of the hypothesis that osteolathyrogenic agents act by blocking aldehydic crosslinking in collagen(5).

Summary. A new series of compounds, β-hydrazinopropionitrile (BHPN) and certain of its methyl derivatives, have been found to have osteolathyrogenic properties for the rat. They cause skeletal deformities (spinal curvatures, periosteal new bone formation), increased saline-soluble collagen levels in sponge biopsy tissue, and occasional mild damage to the aortic media. Neither ruptured aortae nor gross dissecting aneurysms were observed. The most potent lathyrogens of this series are BHPN itself and α-methyl-BHPN. They appear to be comparable in lathyrogenicity to BAPN, but are more toxic than the latter. Less potent was β-methyl-BHPN with N^1-methyl-BHPN being the least potent. N^2-methyl-BHPN (methyl on terminal N of hydrazino group) was not tested.

Addendum. Although we are still unable to prepare any N^2-alkyl-BHPN's, we have recently prepared the N^2-acetyl-BHPN by acetylating BHPN with either acetyl chloride or with acetic anhydride. Chemical tests and IR spectra confirm acetylation on the terminal hydrazino nitrogen. Treatment with HCl converts this compound to aminopyrazoline hydrochloride with the loss of acetic acid. The N^2-acetyl-BHPN is lathyrogenic in our tests and seems to have roughly the same potency as the N^1-methyl-BHPN described above.

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Anserine, A Source of 1-Methylhistidine in Urine of Man.* (29950)

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The amount of 1-methylhistidine excreted in human urine is not influenced by administration of histidine(1) but may be increased when large quantities of meat protein are ingested(2,3,4). When rabbit meat was fed to 2 human subjects an increased excretion of anserine and/or methylhistidine resulted(5).

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