this laboratory. The reaction of this cell line to infection provides a reliable and convenient means for titration of viral infectivity and assay of specific virus-neutralizing antibody.

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Osteolathyrism in the Hamster: Pathological and Chemical Changes Produced by β-Aminopropionitrile in Connective Tissues.* (29764)

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The administration of β-aminopropionitrile (BAPN), a potent lathyrogen, has been shown to increase the soluble collagen fractions obtainable from chick embryos(1), from mammalian skin(2,3) and from sponge biopsy connective tissue from rats(4). Since a soluble component of elastin has been reported by Adair *et al*(5), it seemed of interest to determine whether its level also can be affected by BAPN, especially since lathyrogens are known to damage the medial elastic fibers in aortae of growing animals(6-8). Because we have never been able to find any elastin in sponge implant tissue from rats and because the cheek pouch of the hamster has such great extensibility and apparent elasticity, we have studied the effect of BAPN on the growing hamster, especially its effect on collagen and elastin fractions of the pouch.

Materials and methods. Male, Golden Syrian hamsters, 18-28 days of age, were fed finely milled Rockland Rat Stock Diet containing varying amounts of added BAPN fumarate. Unsupplemented Stock Diet was given control groups. All diets were fed *ad libitum*. The animals were sacrificed after varying periods and their mandibles and femurs were examined for gross skeletal lesions. The aorta and one cheek pouch from each animal were dissected out for microscopic examination, fixed in neutral formalin, embedded in paraffin, sectioned and stained with Hornowski's combined elastic and Van Gieson stains. The second cheek pouch was removed and frozen until chemical fractionation could be undertaken (1 to 8 days).†

Soluble and insoluble collagen fractions from cheek pouches were obtained by modifications of methods used by Levene and Gross (1), Jackson(9) and Dasler *et al*(4). The elastin-like fractions were obtained by an adaptation of the procedure of Adair *et al* (5). All 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(10). The hy-

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† Pouches frozen longer than 8 days were not used. We have found that rat sponge biopsy tissue yielded consistent soluble collagen values when the sponges were frozen up to 8 days.

droxyproline content was considered to be indicative of collagen or elastin-like material in the fractions.

Procedure for obtaining collagen and elastin-like fractions. Pouch pieces were suspended in extracting solutions by means of a Vortex Jr. mixer. In steps 1 through 4, extractions were made at 2°C and centrifugations were carried out at 2900 *g* for 20 minutes in a IEC refrigerated PR-2 centrifuge.

1. The *frozen* pouch was cut into small pieces with scissors and the pieces were suspended in 2 ml of water containing 1 drop of octanol-1. They were extracted 1 hour, centrifuged and the supernate discarded.

2. Pouch pieces were suspended in 3 ml of 0.2 *M* NaCl, extracted 1 hour, centrifuged and the supernate discarded.‡

3. Pouch pieces were extracted overnight with 3 ml of 1 *M* NaCl (pH 7.6 with 0.01 *M* potassium phosphate buffer), centrifuged and the supernate retained. The pieces were extracted twice more for 2-hour periods with 2 ml of 1 *M* NaCl, centrifuged and the supernates retained. The 3 supernates were combined and represent the 1 *M salt soluble collagen fraction.*

4. Extractions of step 3 were repeated using 0.2 *M* citrate buffer, pH 3.5. The combined extracts represent the *citrate soluble collagen fraction.*

5. Pouch pieces were autoclaved 3 times at 15 lb (121°C) in distilled water as follows: in 3 ml for 3 hr; in 2 ml for 1 hr; in 2 ml for 1 hr. They were centrifuged at room temperature in a clinical centrifuge after each treatment. The combined supernates represent the *autoclaved collagen fraction* (insoluble collagen fraction).

6. Pouch pieces were extracted in a boiling water bath for 1-hour periods as follows: with 3 ml of 0.05 *M* oxalic acid; with 2 ml of water; with 2 ml of water. Extractions were centrifuged as in step 5. The combined supernates were neutralized with calcium chloride and filtered and represent the *soluble elastin-like fraction.*

7. Pouch piece residues were retained and represent the *insoluble elastin-like fraction.*

‡ This fraction would not be discarded when tissues which contain 0.2 *M* NaCl-soluble collagen are used.

Results and discussion. Wegelius *et al*(11) found no gross skeletal abnormalities in young adult hamsters injected with the lathyrogen, aminoacetonitrile. In our experiments we found the skeletons of growing hamsters to be much more resistant to the lathyrogenic action of BAPN than those of rats. Thus, when 0.2% BAPN fumarate was fed, the mandibles and femurs were indistinguishable from normal specimens. This level causes marked skeletal abnormalities in the growing rat within one week. When sufficiently high levels of BAPN were fed, however, skeletal abnormalities were produced also in the hamster. The difference in the effect of BAPN on the skeletons of hamsters and of rats is illustrated in Fig. 1.

Although 2 hamsters fed BAPN died from ruptured thoracic aortas, no gross aneurysms such as are commonly seen in rats on such diets were noted in sacrificed animals. Microscopically, medial elastic fiber damage, although present in some animals, was less common and much less severe than in rats fed comparable levels of BAPN.

The normal hamster cheek pouch was found microscopically to consist of an epithelial layer, a connective tissue layer containing collagen-like fibers but no stainable elastin, and a layer of muscle fibers. The loose areolar tissue surrounding the pouch contained fibers which took the elastic stain. No conclusive differences in the staining characteristics for collagen or elastin were found when normal pouches were compared to those from animals fed BAPN fumarate at levels up to 1%.

Chemical fractionation of cheek pouches. No hydroxyproline was found in water extracts of pouches from hamsters on either experimental or control diets. We have found this to be true also of water extracts of sponge biopsy tissue from rats.§

Hydroxyproline was not found in 0.2 *M* NaCl extracts of pouches from hamsters, even from animals fed levels of BAPN fumarate as high as 2%. This situation differs

§ Pre-extraction with water does appear to remove tyrosine-containing material from pouches and sponge biopsies. Tyrosine interferes in the analysis for hydroxyproline(10).

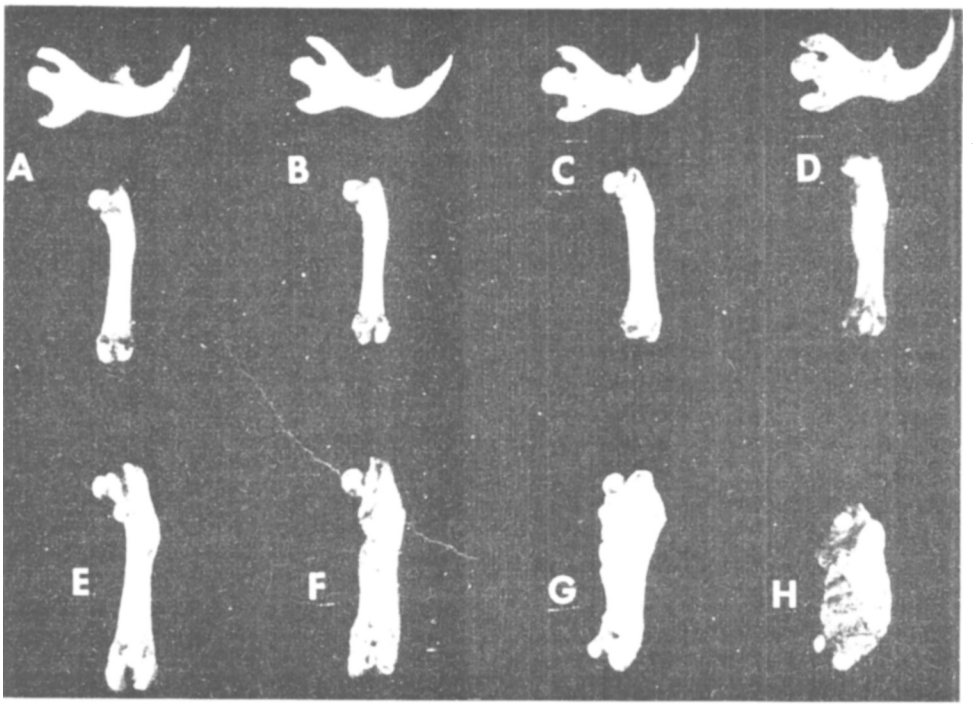

FIG. 1. Skeletal effects of BAPN in hamsters compared to rats. % = % BAPN fumarate in diet; da = days on experiment; ( ) = initial and final weights of animals. *Top row* (hamster femurs and mandibles): A = 0%, 29 da (30-79); B = 0.2%, 22 da (38-63); C = 0.4%, 15 da (40-68); D = 1.0%, 22 da (61-59). *Bottom row* (rat femurs): E = 0%, 23 da (47-192); F = 0.1%, 17 da (57-169); G = 0.25%, 21 da (51-100); H = 0.8%, 14 da (58-66).

from that found in the sponge biopsy tissue of the rat(4). In the latter a small amount of hydroxyproline is found in 0.2 *M* NaCl extracts of normal tissue with a markedly higher level in tissue from rats fed BAPN. The absence of this fraction in the hamster pouch may be associated with the relative resistance of the hamster to skeletal and aortic damage by lathyrogenic agents.

Hydroxyproline indicative of soluble collagen appeared either in *M* NaCl extracts or in citrate extracts of pouches from both normal and lathyritic hamsters but not in both fractions. Thus, when *M* NaCl was used, no soluble collagen was found in the citrate extracts; when *M* NaCl was not used in the extraction procedure, the soluble collagen appeared in the citrate fraction. These two fractions of the hamster pouch have different solubility properties from corresponding collagen fractions of rat sponge biopsy tissue. In the latter, both the *M* NaCl and the citrate fractions from the same sponge have been found by us to contain hydroxyproline.‖

Whether the soluble collagen of the pouches was extracted with *M* NaCl or with citrate, its level was always higher in extracts of pouches from hamsters fed BAPN than in extracts of pouches from normal animals (Tables I and II). This finding may be correlated with the reported decreased tensile strength of healing wounds in aminoacetonitrile-injected hamsters(11).

Since the elevation of the soluble collagen fraction appeared to be relatively independent of the level of fed BAPN (Table I), a time study was carried out with weanling hamsters using the relatively low dietary level of 0.1% BAPN fumarate (Table II). At this level, which induces no gross skeletal changes in the hamster, an elevation of *M* NaCl-soluble collagen was evident in pouches after

‖ For example 14 normal 45 mg sponges implanted 19 ± 4 days gave hydroxyproline contents averaging 68 μg/sponge in the *M* NaCl fraction and 42 μg/sponge in the citrate fraction.

TABLE I. Effect of BAPN on Hydroxyproline in Citrate Extracts of Hamster Pouches.

% BAPN fumarate in diet	Days on diet	Initial body wt (g)	Final body wt (g)	Hydroxyproline (μg/g of pouch*)
2	9	65	59	301
1	16	63 (57)†	69 (78)†	288 (75)†
1	22	61 (61)	59 (85)	413 (117)
.4	15	40 (32)	68 (64)	485 (188)
.4	27	38	59	414
.4	29	68 (66)	95 (100)	289 (175)
.4	42	70 (67)	109 (117)	322 (117)
.1	22	32 (26)	75 (75)	290 (169)
.1	36	37 (30)	80 (79)	319 (163)

* Wet weight. † ( ) = values for control animals.

TABLE II. Effect of Feeding 0.1% BAPN Fumarate on Hydroxyproline in Fractions Extracted from Pouches of Hamsters.

Days on diet	1	4	7	14	21
Wt gain (g)*					
BAPN	3.5	10.0	17.0	28.8	42.5
Normal	3.5	8.5	17.2	29.3	41.0
Pouch wet wt (mg)					
BAPN	290.4 (2)†	281.4 (2)	282.1 (4)	322.6 (4)	322.5 (4)
Normal	234.4 (2)	243.6 (2)	262.9 (4)	295.6 (4)	313.6 (4)
Hydroxyproline					
M NaCl Fraction (μg/g pouch wet wt) (measure of soluble collagen)					
BAPN	200	302	326	408	378
Normal	111	168	179	159	162
Autoclaved Fraction (μg/mg pouch wet wt) (measure of insoluble collagan)					
BAPN	6.09	6.21	6.65	8.87	9.64
Normal	6.39	6.72	6.88	9.22	10.3
Residue (μg/g pouch wet wt) (measure of total elastin)					
BAPN	112	142	344	226	391
Normal	123	156	316	247	447

* Initial weights of hamsters were 30 to 40 g.
† ( ) = Number of pouches averaged.

only one day of feeding and reached a maximum value after about 1½ to 2 weeks on the diet. This elevation thus appears to be a rapid and sensitive indicator of lathyrogenic effects on *M* NaCl-soluble collagen.

Insoluble collagen, *i.e.*, the hydroxyproline-containing material obtained by autoclaving extracted pouch pieces in water, appeared to be slightly lower in extracts from pouches of hamsters fed BAPN. This was correlated with a higher pouch wet weight in the BAPN-fed animals (Table II).

The amounts of hydroxyproline in the oxalate-soluble and oxalate-insoluble elastin-like fractions fluctuated widely and independently of the diet and are therefore not tabulated. The sum of these 2 fractions (*i.e.*, hydroxyproline in the residue) gave much more consistent results and appeared to increase in the pouches with time. This residue fraction appeared to be similar to elastin¶ in that it resisted gelatinization, contained hydroxyproline, and completely dissolved in hot 0.25 *M* oxalic acid after repeated extraction with this solvent(5). No effect of BAPN on this elastin-like material could be demonstrated (Table II).

Summary. BAPN, when fed to weanling male hamsters, produced gross skeletal lesions characteristic of mild osteolathyrism. Aortic damage was also present, but, like the gross skeletal lesions, was much less severe than in rats fed corresponding levels of BAPN. No

¶ An alternative possibility is that this elastin-like material may be collagen that resists solubilization by autoclaving. For a discussion of the difficulties in obtaining collagen-free elastin see Partridge(12).

microscopic changes in the cheek pouches of lathyritic animals were observed. BAPN caused an increase in the *M* saline-soluble collagen fraction or in the citrate-soluble collagen fraction of the cheek pouches. An increase in the *M* NaCl-soluble fraction was evident after only 1 day on 0.1% BAPN fumarate. This increase in the *M* NaCl fraction may be of value in rapid screening of potential lathyrogens. No 0.2 *M* NaCl-soluble collagen fraction could be found in pouches from either normal or lathyritic hamsters. Its absence may be associated with the relative resistance of the hamster to BAPN. No effect of BAPN on elastin-like fractions of the pouches could be demonstrated.

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Inhibition of Influenza C Virus by *Hemophilus influenzae* in Embryonated Eggs.* (29765)

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Previous publications from this laboratory (1,2) have described certain aspects of the bacterial population dynamics when *Hemophilus influenzae,* type b is superimposed on an established Influenza C virus infection in embryonated eggs. Infection with the virus promotes conditions favorable to an accelerated bacterial growth rate and also provides the requirements for maintaining a high proportion of virulent members in the bacterial population. During the course of these studies it became apparent that active infection with *Hemophilus* exerted an inhibitory or depressing influence on the production of virus in a concurrent Influenza C infection. This report concerns results of one series which represents several preliminary experimental studies of this phenomenon.

Materials and methods. Influenza C, strain J. J. maintained in passage by intra-amniotic inoculation of 10 or 11 day embryonated eggs constituted the viral component. A pool composed of bacteria free amniotic fluids collected from the 221st passage with a HA (hemagglutination) titer of 1:512 provided the initial inoculum. *Hemophilus influenzae,* type a, American Type Culture Collection No. 9237 served as the bacterial component. Type a was chosen because it is much less lethal for embryonated eggs than types b and d. Embryonated eggs were prepared by the "window" method during the 11th incubation day. All inoculations were made by the intra-amniotic route by means of tuberculin syringes and 1½ inch long 24 gauge needles.

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