Pitney(6). The unsupplemented chickens continued to excrete large amounts of FGA. Both Vit. B_{12} and the DBC coenzyme caused a marked reduction in FGA excretion, which was down to minimal levels by the end of 2 days. The daily 100 μg Vit. B_{12} supplement, which was given to *all* chicks in the second experiment, caused a very marked and immediate drop in FGA excretion of Vit. B_{12}-deficient chicks that had not been previously supplemented. This very high supplement of Vit. B_{12} also caused a further reduction of FGA excretion by the 2 previously supplemented groups. Thus, by both parameters, growth and reduction of FGA excretion, Vit. B_{12} and DBC coenzyme had similar activities.

Summary. Vit. B_{12} and the 5,6-dimethylbenzimidazolylcobamide coenzyme were equally active in (1) supporting growth of chicks up to 4 weeks of age and in (2) overcoming the impaired metabolism of formiminoglutamic acid that occurs in Vit. B_{12}-deficient chicks.

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Fragility and Extractable Collagen in the Lathyritic Chick Embryo. An Assay for Lathyrogenic Agents.* (26040)

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The lathyrogenic substances, β-aminopropionitrile and semicarbazide, when introduced into the chick embryo induce a loosening of the intermolecular structure within collagen fibrils allowing them to dissolve in cold neutral saline(1). This intrafibrillar alteration is associated with marked increase in fragility of the entire organism. Both phenomena are demonstrable between one and 3 hours following a single injection and increase steadily for at least 72 hours. Both were also shown to be dosage dependent. Analytical studies(1) plus histologic and electron microscope examination of thin sections of normal and lathyritic skin before and after extraction(2) led to the conclusion that insoluble fibrils, formed prior to administration of the agent, were rendered soluble in cold saline. Skin, bone, aorta and tendon were all involved.

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It is the primary purpose of this report to establish the basis for a bio-assay for lathyrogenic activity utilizing induced chick embryo fragility and extractability of collagen.

Procedures. Groups of 20 to 30 fertilized eggs of the white Leghorn variety were injected at 14 days of incubation with a variety of agents dissolved in 0.2 ml of sterile saline. Application was made through a pinhole onto the chorioallantoic membrane as described previously(1). After 48 hours of further incubation embryo fragility was measured, and tissues prepared for extraction.

Mortality rate for all doses was no higher than that for controls, about 10%.

Fragility was measured by determining the weight required to detach the head from the body within a period of 10 to 100 seconds. Three to 5 embryos from groups of 20 were used in preliminary estimates of the proper weight. A more reproducible but somewhat longer procedure involved a continuous load increase to the breaking point, thus eliminating the time factor. This is accomplished by flow of water at a fixed rate into a cellophane bag suspended from the head of the embryo. A useful fragility index was found to be the ratio of the breaking weight for controls to that for experimental embryos.

A biochemical index of activity was obtained by measurement of viscosity and hydroxyproline content of cold saline extracts of minced bones pooled from 10 to 20 treated embryos. Tibiae and femora were stripped of muscle, tendon and periosteum, minced with scissors and extracted in 3 volumes (v/w) of 1 M cold saline containing phosphate 0.02 ionic strength pH 7.6 at 5°C for 24 hours with shaking. The extracts were centrifuged at 100,000 g in the Model L Spinco preparative ultracentrifuge for 1 hour. Supernatant solutions were passed through fine sintered glass filters and viscosity was measured in Ostwald viscosimeters with a flow time of 60 seconds at 5°C. Each extract was analyzed for hydroxyproline and in one experiment (Table I) hexosamine and tyrosine were also determined as described previously(1). Standard curves were obtained using 1, 3, 5, 10, 20 and 30 mg of BAPN fumarate. As a routine only 3 and 10 mg points were used.

TABLE I. Dosage Dependence of Bone Extract Properties and Embryo Fragility.

Dose	Fragility index	η rel	μg/ml of extract		
			Hypro	Hexosamine	Tyrosine
Saline	1.0	1.5	17.0	74.6	240
BAPN fumarate					
2 mg	1.9	3.3	46.0	91.0	314
5	2.5	8.4	122.5	76.3	308
10	5.0	20.0	285.0	80.4	312
20	7.7	23.2	385.0	87.0	310
30	9.1	22.0	390.0	78.8	300

The following substances§ were tested in this series: water, β-aminopropionitrile fumarate (BAPN), aminoacetonitrile, methylene aminoacetonitrile, methylaminoacetylnitrile, semicarbazide, β,β'-iminodipropionitrile, β-alanine, β-mercaptoethylamine, cystamine, taurine, methylamine and cyanoacetic acid.

Iproniazid phosphate (1-isonicotinyl 2-isopropyl hydrazine),‖ an inhibitor of monoamine oxidase, said to enhance lathyrogenic activity of BAPN in the turkey poult(3) was applied as described above in doses of 10^{-2}, 10^{-4}, and 10^{-6} moles, alone and with 3 mg of BAPN.

Results. Data from a representative experiment are shown in Table I. Dose relationships for fragility index and extract viscosity and hydroxyproline were nearly linear between 1 and 10 mg of BAPN and parallel each other. Maximum effect was obtained at about 20 mg. Tyrosine and hexosamine content of the extracts were elevated to about the same degree at all doses.

Table II reports the effectiveness of a number of analogues and substances of known lathyrogenic activity in the rat, as compared to β-aminopropionitrile in equimolar amounts.

Iproniazid alone produced none of the stigmata of lathyrism in the chick embryo, although it increased mortality in doses of 10^{-2} moles. It did not significantly enhance the effectiveness of BAPN. Maximum dose used was much greater than those said to completely inhibit liver monoamine oxidase in the rat and guinea pig(4).

§ Courtesy of Abbott Laboratories.

‖ Courtesy of Roche, Inc.

TABLE II. Relative Activity of Compounds.

Agent	Molar equivalents of BAPN in terms of: Fragility	Viscosity	Hydroxyproline
$NH_2CH_2CH_2CN$ (β-aminopropionitrile)	1.0	1.0	1.0
NH_2CH_2CN (aminoacetonitrile)	2.5	2.5	4.0
$NH \cdot CONHNH_2$ (semicarbazide)	.3	.3	.3
$NH_2 \cdot SC \cdot NH \cdot NH_2$ (thiosemicarbazide)*			
$CH_2{=}NCH_2CN$ (methylene aminoacetonitrile)	2.0	2.5	4.0
$CH_3NHCOCN$ (methylaminoacetylnitrile)	0	0	0
$NH(CH_2CH_2CN)_2$ (β,β'-iminodipropionitrile)	0	0	0
$NH_2CH_2CH_2SH$ (β-mercaptoethylamine)	0	0	0
$(NH_2CH_2 \cdot CH_2S)_2$ (Cystamine)	0	0	0
$NH_2CH_2CH_2SO_2H$ (taurine)	0	0	0
$NH_2CH_2CH_2COOH$ (β-alanine)	0	0	0
$HOOC_2CH_2CN$ (cyanoacetic acid)	0	0	0
NH_2CH_3 (methylamine)	0	0	0
$NaOOC \cdot CH{=}CH \cdot COONa$ (sodium fumarate)	0	0	0

* Thiosemicarbazide was highly active in a dose of 25 mg but was not assayed quantitatively.

Discussion. Embryo fragility and appearance of extractable collagen were always associated following application of lathyrogenic compounds. Dosage dependence for both phenomena were sufficiently close to linear for the range 1 to 10 mg of BAPN to provide a useful quantitative measure for comparison of the effectiveness of various agents.

Analyses for hexosamine, a measure of aminosugar containing compounds such as glycoproteins and mucopolysaccharides, and for tyrosine, a rough measure of non-collagenous proteins indicated no correlation with dosage in either case. However, actual isolation of the large sugar-containing substances is required for any satisfactory evaluation of changes in relative amounts or properties of such molecules.

In practice an unknown compound may be assayed by comparing its effectiveness to that of two different doses of BAPN fumarate, for example 3 and 10 mg (using the same batch of eggs), the comparison being made on a molar equivalent basis.

Among the groups of agents examined in this study, only 5, known to be lathyrogenic in the rat, proved to be effective, namely β-aminopropionitrile, aminoacetonitrile, methylene aminoacetonitrile, semicarbazide, and thiosemicarbazide.[3] β - mercaptoethylamine and cystamine are said by Dasler *et al.*(5) to produce lathyrism in rats, a conclusion disputed by Ponsetti *et al.*(6). These compounds proved to be inactive in the chick embryo in the doses used (9 and 18 mg, respectively). The reported enhancement of lathyrogenic activity by iproniazid(5), a blocking agent for monoamine oxidase, also could not be confirmed in this system.

The series of agents reported is still too small to permit assessment of important common denominators.

We suggest that physical chemical changes such as increased tissue fragility and extractability of collagen are more sensitive and measurable indices of lathyrogenic activity than are morphologic changes, that they represent a phase of the process closer to the biochemical defect, and that their measurement provides a useful tool for deeper exploration of pathogenetic mechanisms.

Summary. A quantitative assay for lathyrogenic agents utilizing induced fragility and extractability of collagen from bones of the chick embryo is reported. Both parameters are dosage dependent and parallel each other. The method has merits of ease, rapidity, reproducibility, and single dose requirement. Fifteen compounds consisting of 5 known lathyrogens and a number of analogues were assayed. Iproniazid phosphate had no enhancing effect on lathyrogenic activity. No correlation between dose and extractable hexosamine and tyrosine were found.

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Comparative Metabolic Studies of H^3 and C^{14} Labeled Stearic Acid.* (26041)

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With the introduction of liquid scintillation spectrometers, beta-emitting tracers began appearing more frequently in studies using radioisotopes. Tritiated compounds have been thought advantageous because of ease in preparation, high specific activity, low cost, and relative safety. However, we have often considered whether H^3 might also be fraught with properties that do not lend it to accurate tracing of specifically labeled fatty acids. With these thoughts in mind, the following investigation was undertaken to compare metabolism of H^3 and C^{14} labeled stearic acid.

Materials and method. Tissue absorption studies. Approximately 0.6 mg of stearic-1-C^{14} and 0.15 mg of stearic-9, 10-H^3 (5-10 μc) were mixed in 1 cc of corn oil and fed by means of stomach tube into male Sprague-Dawley rats weighing 300-450 g. They were maintained on a diet of Purina Fox Chow before and during experiments. Animals were decapitated at intervals of 15, 30, 45, 60, 90, 120, 180, and 240 minutes. Livers and epididymal fat pads were excised and homogenized in methanol or immediately frozen at −15°C. Tissue lipids were extracted with chloroform-methanol as described by Van Handel and Zilversmit(1). Five milliliter aliquots of the extracts were then evaporated and placed in counting solvent, consisting of 0.4% PPO† and 0.01% POPOP‡ in reagent grade toluene. Counts were made in a Packard Tri-Carb Liquid Scintillation C-14 Spectrometer. Simultaneous equations were then used to determine the separate values contributed by each of the 2 labels when both were counted simultaneously. Counting at HVT 5, window 10-80, efficiencies of around 50% for C^{14} and 0.3% for H^3 were obtained; while at HVT 9, window 10-50, efficiencies of around 11% for C^{14} and 15% for H^3 were obtained. Thirty-two animals were studied in this manner. *Lipid fraction studies.* In these analyses the dose was raised to 50 μc per type of stearic acid so as to increase sample specific activity. Aliquots analyzed by the silicic acid columns were reduced to 0.0125 cc for fat and 0.025 cc for liver compared to the 5 cc aliquots used in the absorption studies. Animals were decapitated 4 hours post injection, liver and fat pad were weighed, then immediately placed in the homogenizer. Chloroform-methanol extracts were further processed to remove protein contamination by the method suggested by Enteman(2), using a flash evaporator at 60°C, successive chloroform-methanol extractions, and filtration through fat-free filter paper. The lipid obtained was then fractionated on silicic acid columns according to the method of Hanahan (3). To check the manner in which differently labeled fatty acids were handled by the silicic acid columns, we put known concentrations of stearic-1-C^{14} and stearic-9, 10-H^3 on the columns and then carried out the usual elution procedure.

Results. Liver and epididymal fat pad absorption. Variation when comparing H^3 to C^{14} in specific activity of the labels in each tissue is apparent. Variations of similar magnitude were found in the counts of the same type labeled stearate in different animals killed at the same time (Table I). On some

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† PPO or 2, 5-diphenyloxazole.

‡ POPOP or p-bis (2-(5-phenyloxazole))-benzene from Pilot Chemicals, Inc., Watertown, Mass.