liminary experiments, 110 cells from a tissue culture of 67% viability have been introduced to grow very satisfactorily in the medulla of the femur.

From our results it would seem reasonable to suggest that in the absence of parathyroid glands the animals are more resistant to tumors invading bone than littermates with intact parathyroids. The place of parathyroid hormone in malignant invasion of bone remains to be clarified.

Summary. Fibrosarcoma induced in the pituitary by feeding 3-MC to a castrated male Sprague-Dawley rat, has been transplanted into the medullary cavity of the femur in female animals. Growth progress within the bone has been successfully demonstrated by X-rays. Parathyroidectomy prior to introduction of the tumor significantly prolongs the survival time of the animal.

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Effect of Two Lathyrogens on Cytochrome Oxidase in Cultures of the Strain L Fibroblast.* (29709)

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Osteolathyrism, a toxic syndrome involving the connective tissues of experimental animals, can be produced by a variety of chemical compounds. The pathogenesis of the disorder has been the subject of investigation for several years. One theory proposes that the disorder involves inhibition of cytochrome oxidase, an enzyme mediating transfer of electrons to molecular oxygen in the major chemical oxidative energy pathway of cell mitochondria(1). Aminoacetonitrile (AAN) and beta-aminopropionitrile (BAPN) are the 2 most widely used lathyrogenic compounds. Each has been shown, using different systems, to be capable of inhibiting cytochrome oxidase(1,2). The present experiment is an attempt to appraise the relative efficacy of these 2 substances as enzyme inhibitors in comparison with their relative efficacy as lathyrogens under the comparatively constant conditions of an *in vitro* tissue culture system.

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Material and methods. Cells of the L strain, originally derived from mouse fibroblasts, were used in this experiment. The growth medium consisted of Eagle Minimum Essential Medium(3) (Hyland Laboratories) to which was added 10% calf serum, 1% L-glutamine, and antibiotics (penicillin and streptomycin).

Di-BAPN fumarate, AAN hydrogen sulfate, and sodium fumarate solutions were each prepared in minimum essential medium and neutralized with one normal sodium hydroxide to pH 7. Sterilization was achieved by millipore filtration.

L cells from 4-day-old stock cultures were resuspended in fresh growth medium, to which BAPN, AAN or the fumarate control solution had been added. The suspended cells were pipetted in 4.0 ml aliquots into sterile one-ounce rectangular wide-mouth bottles containing glass cover slips. These were then sealed and incubated at 37°C for 72 hours.

TABLE I. Outline of Experimental Procedure.

	NADI medium					
Culture medium	Control	Control fumarate	Fresh AAN	72 hr AAN	Fresh BAPN	14 day BAPN
Control	4 cult.	4 cult.	4 cult.	4 cult.	4 cult.	4 cult.
AAN 5 mmol added	4 "	4 "	4 "	4 "		
BAPN 20 mmol added	4 "	4 "			4 "	4 "

The presence of cytochrome oxidase was assessed by means of the G-Nadi reaction(4), using a solution of 1.16 millimolar alpha Naphthol (Eastman), 1.35 millimolar N,N-dimethyl-p-phenylene diamine monohydrochloride (Sigma, grade II), and 0.155 molar sodium chloride, buffered to pH 7.4 with Sorensen phosphate buffer system. BAPN 20 millimolar, AAN 5 millimolar or sodium fumarate 10 millimolar was added to the Nadi reagent solutions to correspond to the culture conditions (Table I), and sodium azide 5 millimolar was included in one Nadi solution as a known cytochrome oxidase inhibitor for a control negative reaction.

Each portion of the experiment was performed in quadruplicate. The cover slips with attached cells were removed from the incubation bottles after 72 hours and placed in the Nadi reagent solution at room temperature for 30 minutes, briefly rinsed in 0.9% saline solution and mounted on slides with a saturated aqueous solution of potassium acetate. Photomicrographs were taken of representative areas within the 30 to 60 minutes following incubation. Slides prepared by this method were not permanent but were unchanged for at least 24-48 hours.

The ability of this procedure to permit distinction of quantitative differences in cytochrome oxidase activity was tested in one experiment by subjecting 72 hour control cultures to Nadi reagent containing varied concentrations of potassium cyanide: 10^{-7}, 10^{-6}, $10^{-5.5}$, and 10^{-3} molar. The data of Clemmons and Jackson(1) indicate that sodium cyanide causes no inhibition of rat myocardial cytochrome oxidase at a concentration of 10^{-7} molar, 25 to 30% inhibition at a concentration of 10^{-6} molar, 50 to 60% inhibition at a concentration of $10^{-5.5}$ molar, and 100% inhibition at 10^{-3} molar. Cultures subjected to these 4 cyanide concentrations during the Nadi reaction could be readily distinguished from each other. Inhibition of the order to 25% or greater should therefore be visually identifiable, in comparison with controls, using this technique.

Results. Cells grown in the medium containing 20 millimolar BAPN and those grown in medium containing 5 millimolar AAN showed a definite growth inhibition and/or failure to adhere to the cover slips (Fig. 1). Fig. 2 is a fumarate control culture. Cover slips from each group showed a reduction in cell concentration to approximately one-third of control culture cell concentration. This is in general agreement with the concentrations of these reagents shown to exhibit cytotoxic effects in tissue culture fibroblasts by several investigators(5,6,7). The cytotoxicity produced with these concentrations of the two lathyrogens was of approximately equal degree. This is in agreement with the relative potency of the 2 compounds in another study(8).

In neither the BAPN or AAN cultured cells, however, could any inhibition of cytochrome oxidase activity be shown when they were removed from the lathyrogenic medium, rinsed briefly, and immersed in a control Nadi solution. Addition of a freshly prepared solution of AAN or of BAPN to the Nadi reagents in concentrations equivalent to those in the culture media also failed to cause any visible inhibition of cytochrome oxidase (Fig. 3, 4). Use of Nadi reagent containing a 5 millimolar concentration of AAN from an AAN stock solution prepared 72 hours in advance, however, caused virtually complete inhibition of cytochrome oxidase in the cells (Fig. 5). This inhibition occurred in cells grown in control culture media as well as in cells grown in the presence of AAN. No visible inhibition of cytochrome oxidase was produced with BAPN in the Nadi reagent, using stock

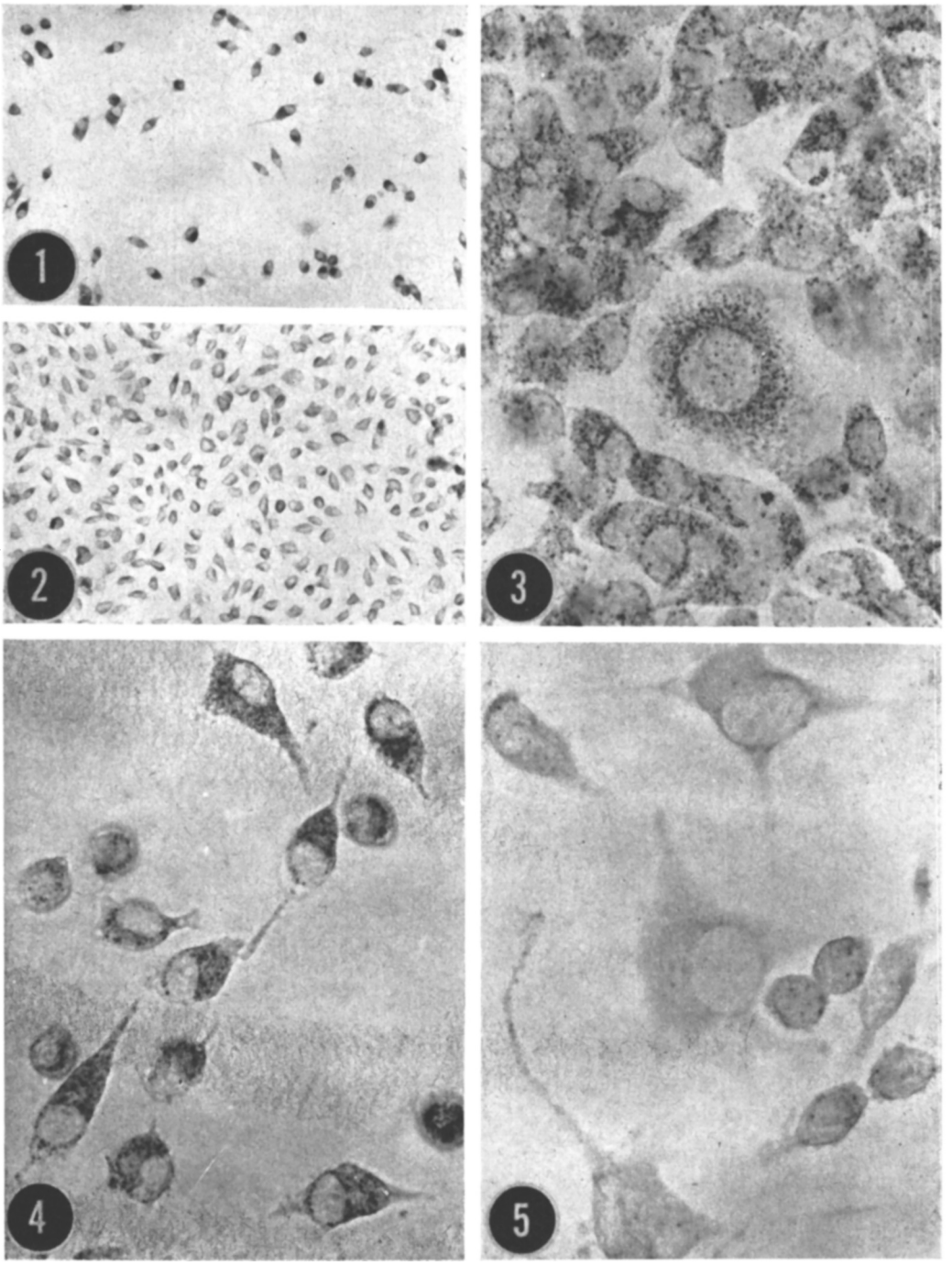

FIG. 1. L-cells cultured 72 hours in medium containing 20 millimolar BAPN. There is marked reduction in the cell concentration. Compare with Fig. 2. Mag. 140 ×.

FIG. 2. L-cells cultured 72 hours in control medium. Mag. 140 ×.

FIG. 3. L-cells cultured 72 hours in control medium, and incubated in control Nadi reagent. The dark cytoplasmic stippling represents deposits of the indophenol blue at sites of cytochrome oxidase activity. The unstained areas of the cells represent nuclei. Mag. 575 ×.

FIG. 4. L-cells cultured 72 hours in medium containing 20 millimolar BAPN, and incubated

in Nadi reagent containing 20 millimolar BAPN. Despite a decrease in cell number when compared with Fig. 3, cytochrome oxidase activity appears to be unchanged. Mag. 575 ×.

FIG. 5. L-cells cultured 72 hours in medium containing 5 millimolar AAN, and incubated in Nadi reagent containing 5 millimolar "aged" AAN. Cytochrome oxidase activity is virtually absent. Mag. 575 ×.

TABLE II. Activity of Cytochrome Oxidase in L-Cells.

		NADI medium			
Culture medium	Control	Fresh AAN	72 hr AAN	Fresh BAPN	14 day BAPN
Control	+	+	—	+	+
AAN 5 mmol added	+	+	—		
BAPN 20 mmol added	+			+	+

BAPN solution 14 days old, and in one trial a stock solution several months old (Table II).

Discussion. Aminoacetonitrile (AAN) in aqueous solution is unstable *in vitro,* as has been demonstrated by Clemmons and Jackson(1). Unaltered (freshly prepared) AAN has no evident inhibitory effect upon cytochrome oxidase. One of the deterioration products in solution, presumably cyanide, exerts a marked inhibitory effect on the enzyme. The relationship of the inhibitory effect of the degradation product to the lathyrogenic capability of this compound is unknown. Demonstration that other lathyrogenic compounds or *in vivo* degradation products inhibit cytochrome oxidase would provide a circumstantial basis for implicating the enzyme inhibition as an etiologic factor in lathyrism. The two effects, enzyme inhibition and lathyrogenicity, should, in addition, have similar proportional relationships among the different lathyrogens.

BAPN failed to produce any discernible inhibition of cytochrome oxidase at a concentration approximately equivalent in potency to concentrations of AAN producing complete inhibition of the enzyme. "Aged" BAPN solutions also failed to inhibit the enzyme. The BAPN concentration used was 15 times higher than concentrations shown by McCallum and Paul to inhibit completely elastica formation in culture explants of chick embryo heart(6). This information, coupled with the marked growth retardation and/or failure to adhere to the cover slips exhibited by the cultures in BAPN, makes it unlikely that inadequate dosage of the BAPN is responsible for failure to exhibit enzyme inhibition.

The marked discrepancy in enzyme inhibitory action of the 2 lathyrogens, at concentrations which are cytotoxic and approximately equivalent, raises considerable doubt as to the role of enzyme inhibition in the production of lathyrism.

Clemmons has recently shown(2), using chick embryos, that BAPN does inhibit cytochrome oxidase partially at a dosage level producing a definite alteration in hydroxyproline metabolism of the embryo. His data, in contradistinction to ours, support, a relationship between cytochrome oxidase inhibition and osteolathyrism. A study of the relative inhibitory potency of AAN and BAPN in his system, and its relation to the potency of the two compounds as lathyrogens, seems warranted.

Summary. An attempt was made in this experiment to assess the proposed relationship of cytochrome oxidase inhibition to the production of lathyrism, an experimental disorder of connective tissue. Aminoacetonitrile, a potent lathyrogen, does not inhibit cytochrome oxidase in cultures of L-cells unless allowed to deteriorate in solution. Beta-aminopropionitrile, a widely used lathyrogen, does not inhibit cytochrome oxidase in cultures of L-cells, and does not appear to yield deterioration products in solution capable of inhibiting the enzyme. This discrepancy in enzyme inhibitory capability, at concentrations of the two compounds which are approximately equivalent lathyrogenically and which exert a cytotoxic effect on L-cells, casts doubt on the proposed relationship.

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A Fatal Septicemic Disease of Infant Puppies Caused by Cytopathogenic Organisms with Characteristics of *Mycoplasma.** (29710)

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In many breeding kennels there may occur sudden unexpected neonatal or infant puppy deaths. During an investigation of such unexplained deaths, there were brought to our attention 2 similar occurrences in breeding kennels located in central New York, in each of which young puppies had died from what appeared to be a septicemic disease that could not be attributed to recognized viral, bacterial, or protozoan infections. From dead puppies that were submitted for study, agents were isolated and grown to tissue culture. These proved cytopathogenic for dog kidney cell cultures (DKC) and possessed characteristics of *Mycoplasma*(1,9). In another instance DKC cultures degenerated spontaneously and from them a similar cytopathogenic agent was isolated. This report concerns some of the characteristics of these agents and certain features of this apparently new disease of puppies.

Materials and methods. Source of isolates. (1) An agent cytopathogenic for DKC cultures was isolated in Nov., 1961 from the heart blood, lungs, livers, spleens, and kidneys of 3 Springer Spaniel puppies that died between 2 and 3 weeks of age. All the remainder of a litter of 12 also died. The only sign of illness had been continual crying that began 8 to 12 hours before death. A kidney isolate, designated strain F-205, was selected for further study. (2) A second isolate was obtained in August, 1962 from DKC cultures that originated in Illinois. A history of the puppies that donated the kidneys for cell culture was not available. Because spontaneous cytopathic effects (CPE) resembled those caused by certain viruses, cultures were submitted to the Veterinary Virus Research Institute for study. From them a cytopathogenic agent was isolated and was designated strain A-1. (3) In Oct., 1963 agents cytopathogenic for DKC that produced CPE similar to those caused by strains F-205 and A-1 were recovered from lung, kidney, spleen, and lymph node tissues from 3 littermate Beagle puppies that had died within 2 to 3 weeks after birth. These specimens were brought to us within a few hours after death and from them a spleen isolate, designated T-O, was selected for further study.

Portions of tissues from various organs were selected from all of these puppies at time of necropsy. They were cultured both aerobically and anaerobically on horse blood agar and stored at —60°C in a mechanical freezer. Portions also were fixed in 10% neutral formalin or in Bouin's fluid. Later, the stored tissues were triturated in medium 199 (Difco) to make a 10% suspension and inoculated into DKC cultures.

Histopathological examination. Paraffin

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