upon adrenal medullary function reported.

While the mechanism of this control of medullary function by cortical tissue is at present not clearly understood, the findings here are reminiscent of the permissive role of hormones in other systems. Whether or not this type of control constitutes a permissive or a direct effect of adrenal cortical hormones in the regulation of adrenal medullary function is currently under investigation.

Summary. Hypophysectomy in the rat leads to a decrease in adrenal medullary concentrations of phenylethanolamine-*N*-methyltransferase (PNMT), the enzyme which catalyzes the conversion, *in situ*, of norepinephrine to epinephrine. Unlike the immediate decrease of glucocorticoids, this decrement takes several days (weeks) to occur. When ACTH is now given to such animals, not only is steroid synthesis resumed, but there is an immediate activation of PNMT and epinephrine synthesis.

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Collagen Synthesis in Cultured Cells: The Influence of Beta-Aminopropionitrile* (33577)

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Experimental lathyrism is a connective tissue disease exhibiting changes in collagen, elastin and ground substance (1–3). The nature of the defect in collagen has been shown to be a failure in cross-linking which in turn is manifested by an increase in neutral salt soluble collagen in lathyritic animals (4–6). There appears, however, to be no direct *in vivo* effect of lathyrogens on the total synthesis of collagen. Similar *in vivo* studies of the effect of lathyrogens on polysaccharide synthesis have been conflicting with reports in the literature of no change, an increase, and decrease in total synthesis (7–10).

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In vitro studies, however, have consistantly indicated an increase in overall polysaccharide production in cell cultures treated with lathyrogens. There are reports of increased ^{35}S uptake (11) and, increased glycogen and uronic acid synthesis (12) in lathyrus-treated strain L fibroblasts, and an increase in protein bound hexose in fibroblasts isolated from human gingiva (13). The *in vitro* effect of lathyrogens on established collagen-producing cell lines has not been investigated. The purpose of this study, therefore, was to investigate the production of collagen by lathyrogen-treated cells in culture.

Materials and Methods. Culture. The 3T6 fibroblasts, known to produce collagen (14–16), were grown in 60 × 15-mm plastic

petri dishes in the Dulbecco-Vogt modification of Eagle's medium containing 10% calf serum. Five-day-old cultures were trypsinized and resuspended into a common culture having approximately 100,000 cells/ml. The common culture was then divided equally; one designated as experimental to which was added 5 m*M* β-aminopropionitrile fumarate (BAPN) and the other as the control to which was added an equal concentration of sodium fumarate. The dosage of 5 m*M* BAPN was established by assessing the toxicity of various concentrations of the lathyrogen. This concentration produced the maximum response without affecting cell viability.

Replicate cultures were prepared by dispensing 4.5 ml of cell suspension into each culture plate and incubating them at 37°. The medium was changed three times weekly for the duration of the experiment. Cells were harvested at 2, 4, 7, 9, 11, and 14 days of culture. The harvest schedule was such that the 2-, 4-, 9-, and 11-day samples were in contact with fresh medium for 1 day, and the 7- and 14-day samples in contact for 2 days prior to harvest. In all cases, however, all media were pooled and saved so that the results reported represent the total hydroxyproline produced to the day of harvest. The medium was decanted and centrifuged at 200*g* for 10 min to remove any dislodged cells or debris. Ethylenediaminetetraacetic acid (EDTA) 0.02% in a phosphate buffer was then added to the cell monolayer and incubated at 37°. After 10 min the detached cells in the EDTA were again centrifuged and the cells, medium, and EDTA wash were separated for analysis. Quantitation was based on both cell count and DNA analysis (17).

Chemistry. The medium was analyzed for collagen as measured by free, peptide, and protein-bound hydroxyproline. Four vol of cold absolute alcohol were added to 4 ml of medium. After 3 hr at 4°, the mixture was centrifuged and the supernate and precipitate were dried separately. The precipitate was then hydrolyzed in 6 *N* HCl for 16 hr at 104°. The supernate was divided into two

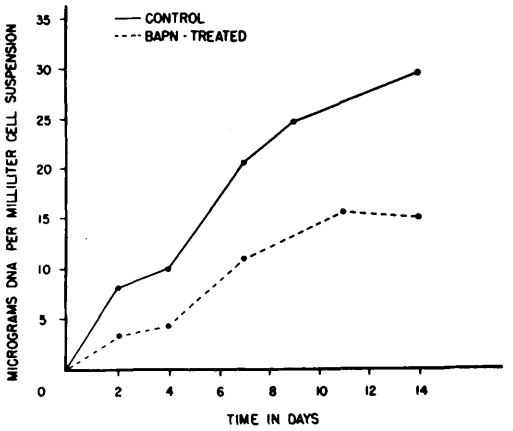

FIG. 1. The effect of 5 m*M* β-aminopropionitrile on cellular proliferation for a 14-day period.

fractions. One fraction was hydrolyzed as above and the other taken up with water. The peptide-bound hydroxyproline was determined by taking the difference between the hydrolyzed and nonhydrolyzed supernate, and that in the original precipitate was designated as protein-bound. The cells were hydrolyzed separately and analyzed for total hydroxyproline. The hydroxyproline was determined by the method of Prockop and Udenfriend (18). Recoveries by this method averaged 86.8 ± 4.2%. All readings were done at 560 mμ on the Hitachi-Perkin Elmer spectophotometer.

Results. Growth. Figure 1 shows the effect of 5 m*M* BAPN on the growth of cells at the various time intervals. Each point represents an average of four cultures. Although growth of cells was depressed by the lathyrogen, the viability was not affected at this concentration. The depression caused by the lathyrogen is similar to that found by others working with other cell lines (11, 12).

An outstanding feature of the experimental cells was the ease with which they could be removed from the culture plates and the uniformity of the resulting cell suspension. The control cells, on the other hand, were rather tenacious, formed a membrane-like material, and were rather difficult to suspend uniformly.

Chemistry. Measurable amounts of hydroxyproline in the medium were found at

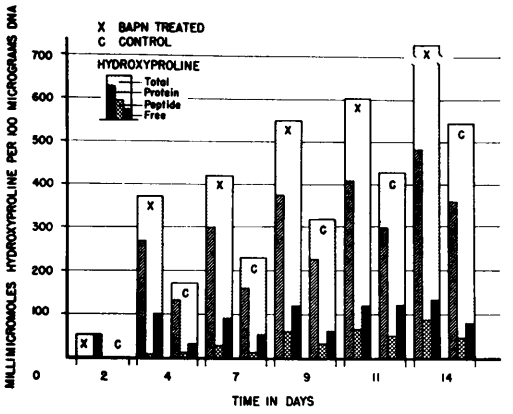

FIG. 2. Collagen production as measured by free, peptide, and protein-bound hydroxyproline over a 14-day period.

every time interval examined except at 48 hr where only the lathyrogen-treated cells produced adequate amounts for analysis. The control cells produced approximately 32.2 mμmoles of hydroxyproline/100 μg of DNA per day while the BAPN-treated cells produced approximately 62.6 mμmoles under the experimental conditions described. The rate of hydroxyproline produced fluctuated depending upon the initial seeding of the experiment and time required for the cells to reach confluency. Although the results were not identical, the difference between experimental and control cells in any of the experiments performed were quantitatively different. Figure 2 represents a typical experiment with an initial seeding of 100,000 cells/ml. Each point represents an average of four cultures. It can be noted that the experimental cells produced almost twice as much hydroxyproline as did the controls, but the ratio of free, peptide, and protein-bound hydroxyproline in both the experimental and control cells did not change appreciably in any of the time intervals examined.

Discussion. The elevated production of hydroxyproline by the experimental cells is in conflict with the reported findings *in vivo* where apparently no difference was found in the total collagen produced by the lathyritic animal when compared with the control (4–6). However, in view of the enhanced production by lathyrogen-treated "L" cells of a sulfate containing large molecule, presumably chondroitin sulfate, reported by Bickley (11), and an increase in mucopolysaccharide-uronic acid reported by Aleo (12), the increase in collagen production may suggest a general stimulative effect, *in vitro*, of BAPN on the synthetic capabilities of cultured cells. Recent reports, however, have indicated that dimethyl sulfoxide, actone, or methanol (19) may also increase the hydroxyproline-producing capacity of fibroblasts *in vitro* so that the effects of BAPN may not be specific. Since the growth of the lathyrogen-treated cultures was suppressed it may be that the depression in proliferation may demonstrate a change in the overall metabolism of the cell from a function of replication to one of the production of extracellular products. This inverse relationship between replication and function has been demonstrated in other systems (20).

It is well documented that, *in vivo*, lathyritic collagen shows an increase in the neutral salt soluble fraction. This defect has been attributed to an alteration in the cross-linking between collagen molecules occurring at both the inter- and intramolecular levels (6). The results in this investigation indicate that the progression of the free amino acid into the peptide and protein-bound fractions is not affected by the lathyrogen. Whether this represents the initial stages of the orderly maturation of collagen which is demonstrated by neutral salt and weak organic acid solubility cannot be ascertained from these data.

Summary. The exposure of 3T6 fibroblasts to the lathyrogen β-aminopropionitrile fumarate caused a reduction in proliferation of the cells but had no effect upon viability. Chemical analysis for the production of collagen as measured by free, peptide, and protein-bound hydroxyproline showed that the lathyrogen exerted a stimulative effect. The lathyrogen-treated cells produced approximately twice the amount of hydroxyproline as did the control cells. The data also indicate that the initial stages of collagen production are not altered and that the depression in proliferation signifies a change in overall

metabolism rather than a direct toxic effect.

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