route. Four viral populations which possessed different combinations of these properties were obtained. In 11 of 12 experiments, one passage in cell culture yielded a virus population (Ty-1) that titered to $10^7$ $MICLD_{50}$/ml on day 5, that failed to cause cell lysis (cpe), that was equally stable at 50% and 80% RH, and that was lethal for monkeys. In contrast, in 4 of 6 experiments, 3 passages in cell culture yielded a virus population (Ty-3) that titered $10^8$ $MICLD_{50}$ or greater on day 3, that induced a cpe, that had a greater sensitivity to 50% than to 80% RH, and that was non-lethal for monkeys. In the remaining experiments, one "atypical" viral population (ATy-1) was obtained from culture after one passage. In 2 other experiments, "atypical" viral populations (ATy-3) that were similar to each other but different from ATy-1 were obtained after 3 passages. The characteristics demonstrated by ATy-1 and ATy-3 suggested that these were intermediate to Ty-1 and Ty-3. The manner in which population characteristics became altered during the conversion of Ty-1 to Ty-3 *in vitro* suggested that some of the characteristics were closely associated with one another.

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## Mucopolysaccharide Defect in Experimental Lathyrism.* (30168)

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The most striking biochemical alteration in the connective tissue of lathyritic animals has been found by several authors to be a change in the solubility of collagen(1,2,3). Not quite the same agreement exists regarding a change in the connective tissue mucopolysaccharides (MPS). While some authors found a decrease of MPS in epiphyseal plates and fracture callus of aminoacetonitrile (AAN)-treated animals(4,5,6), no change whatsoever was found in skin(7). The MPS composition of skin is known to differ considerably(8,9) from that of epiphyseal plate (10) and bone callus(11,12). A possible explanation of the different results obtained in these tissues could be that only certain types of MPS (for ex. Chondroitin-4-$SO_4$ and Chondroitin-6-$SO_4$) are affected by the lathyrogenic agents. In those tissues where these particular MPS are not present or are present only in very small amount, lathyrism would not produce any change in the MPS content. Fracture bone callus has been chosen as a suitable tissue on which to test this hypothesis. Supporting evidence is reported here.

*Experimental.* In 2 experiments performed under the same conditions, the middle third of both tibiae of 40 Sprague-Dawley male rats weighing approximately 160 g were fractured under ether anesthesia. The fractures were not immobilized. The animals were divided in 2 groups one of which was kept on a Purina chow diet and served as control, while the other group received Purina chow containing 0.075% AAN sulfate. Both groups received daily the same amount of

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TABLE I. Hexosamine and Collagen Content of Bone Callus 10 and 17 Days After Fracture.*

| Animals | No. of rats | Days after fracture | Hexosamine, mg/g dry tissue: Glucosamine | Galactosamine | Total | Collagen: Total, mg/g dry tissue† | 1 N NaCl soluble, % of total | 0.5 N $CH_3COOH$ soluble, % of total |
|---|---|---|---|---|---|---|---|---|
| Normal | 10 | 10 | 4.2 | 22.1 | 26.3 | 228 | 2.00 | .23 |
| | 10 | 17 | 2.7 | 12.5 | 15.2 | 203 | 1.47 | .43 |
| Lathyritic | 10 | 10 | 3.9 | 8.4 | 12.3 | 211 | 5.70 | 1.35 |
| | 10 | 17 | 2.6 | 4.7 | 7.3 | 176 | 3.80 | 2.40 |

* Mean values from 2 experiments. All hydrolysis and determinations were done in duplicate.
† mg hydroxyproline × 7.46.

food to avoid a greater increase in weight of the control animals. The animals were sacrificed, one-half at a time, 10 and 17 days after fracture. The calluses of the animals in the same group, after being dissected free from other tissues, were sliced and pooled together. The tissues were kept cold during all these operations.

Samples of tissue from the 2 groups were taken for 1) determination of dry weight by heating at 104°C to constant weight; 2) determination of total hexosamine and hydroxyproline and chromatographic separation of glucosamine and galactosamine; 3) extraction of soluble collagen. Total hexosamine (13) and hydroxyproline(14) were determined after hydrolysis with 4 N HCl for 15 hours at 100°C in sealed ampoules. The same hydrolyzate, after drying and redissolving in 0.3 N HCl, was used for chromatographic separation of glucosamine and galactosamine on Dowex 50 resin according to Gardell(15). For extraction of soluble collagens the tissue was ground with 1 N NaCl (1:10, w/v) in a VirTis 45 homogenizer for 10 minutes, then transferred to round bottom centrifuge tubes. The extraction was continued for 24 hours in a shaking bath at 3°C. The homogenate was centrifuged at 0°C (4000 r.p.m. for 10 minutes). The precipitate was then washed twice in the centrifuge with cold 1 N NaCl. The combined supernatant solutions were centrifuged for one hour at 100,000 × *g* in a Spinco model L. The small amount of sedimented material was discarded and the supernatant solution used for determination of salt-soluble collagen. The residue from the NaCl extraction was resuspended in 0.5 N $CH_3COOH$ and extracted for 24 hours in the shaking bath at 3°C. The acid extracted collagen was purified under the same conditions described above for the salt-soluble fraction. Portions of the clear collagen solutions were hydrolyzed in sealed ampoules at 140°C for 3 hours in 6 N HCl(16) and then analyzed for hydroxyproline(14).

*Results.* The data (Table I and Fig. 1) demonstrate that the hexosamine content of bone callus in the AAN-treated animals is greatly reduced compared to the controls, in agreement with the results reported by Bolognani(6). What we consider the new and most interesting finding is the change in ratio of the 2 aminosugars, galactosamine and glucosamine: a galactosamine/glucosamine ratio of about 5 for the controls as compared to a ratio of about 2 for the experimental animals. The change in ratio is due wholly to a decrease of galactosamine,

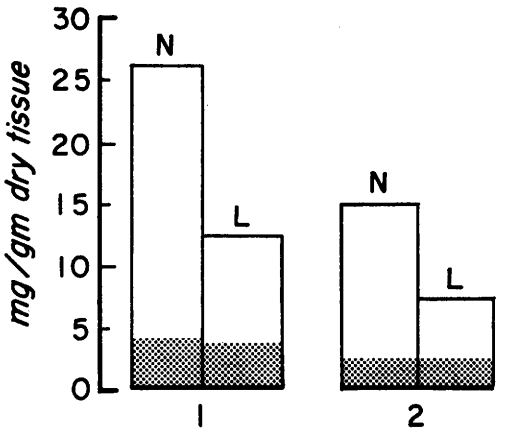


FIG. 1. Glucosamine (dotted blocks) and galactosamine (open blocks) content of bone callus 10 (1) and 17 (2) days after fracture in normal (N) and lathyritic (L) rats.

since the glucosamine content remains the same. Galactosamine, both 10 and 17 days after fracture, is decreased by about 62% in the callus of AAN-treated animals.

Our data confirm also that the total collagen content of fracture callus is not changed in the lathyritic animals, but that its solubility is greatly increased(3).

*Discussion.* Galactosamine is a component of Chondroitin-4-sulfate (Ch-4-$SO_4$), Chondroitin-6-sulfate (Ch-6-$SO_4$) and Dermatansulfate. Ch-4-$SO_4$ and Ch-6-$SO_4$ are the major MPS constituents of bone callus and epiphyseal plate(10,11,12) but occur only in very small amounts in skin, while Dermatansulfate is not present in bone callus and epiphyseal cartilage, but is found, together with hyaluronic acid and heparin in the skin of rats(17). Our data show that in both the 10- and 17-day callus the decrease of hexosamine in lathyritic rats is due entirely to galactosamine. They are in good agreement with those which Castellani *et al* obtained from epiphyseal plates of normal and AAN-treated rabbits(5). Although their results show also a small decrease of glucosamine, it can be calculated from the published data that galactosamine accounts for about 93% of the total decrease of aminosugars in this tissue. It can be concluded that in osteolathyrism there is indeed a defect of MPS metabolism, but this defect is most probably restricted to the two isomeric Chondroitinsulfates (Ch-4-$SO_4$ and Ch-6-$SO_4$).

The deficiency of Ch-(4 or 6)-$SO_4$ might be due to defective synthesis of these two MPS. Available data indicate a lower incorporation of $^{35}S$ in cartilage cells in lathyritic animals(17,18,19,20). A markedly decreased activity of the enzymatic system responsible for the synthesis of hexosamine has also been found in epiphyseal plates of AAN-treated rabbits(21). However, a defect in utilization of these MPS or an excessive removal cannot be ruled out.

Thus, in osteolathyrism it appears that both major components of the ground substance of connective tissue (collagen and MPS) are affected. However, while an increased solubility of collagen, probably due to a deficient intra- and intermolecular cross-linking(22,23) has been found in all types of connective tissue which have been studied in lathyritic animals, a decreased content of MPS has been found only in those tissues which contain large amounts of Ch-4-$SO_4$ or Ch-6-$SO_4$.

*Summary.* The hexosamine content of bone callus of aminoacetonitrile-treated rats has been studied 10 and 17 days after fracture and has been found to be decreased by about 50% as compared to controls. The decrease has been found to be due wholly to galactosamine, while glucosamine content remains the same. On the basis of these and of previously available data it is concluded that only Ch-4-$SO_4$ and Ch-6-$SO_4$ are affected by the AAN intoxication. It has also been confirmed that the solubility of collagen in the lathyritic bone callus is greatly increased.

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## A Metabolic Difference Between Two Lines of Lymphoma 6C3HED Cells in Relation to Asparagine.* (30169)

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Lymphoma 6C3HED cells of Gardner's original line (here designated Lymphoma 6C3HED-OG)(1) have long been carried in this laboratory through repeated transfers—either subcutaneously or intraperitoneally—in C3H mice. These cells are regularly susceptible to the effects of guinea pig serum *in vivo*(2), recent findings having indicated that the asparaginase activity of guinea pig serum is responsible for its antilymphoma effects(3) and that the susceptible cells may depend upon asparagine for growth *in vitro* (4). A subline of 6C3HED cells (here designated Lymphoma 6C3HED-RG1) was obtained several years ago from a mouse given "sub-curative" amounts of guinea pig serum (5). The cells of the RG1 subline are morphologically identical with those of the OG line and they resemble the latter closely in biological behavior, yet they are completely refractory to the effects of guinea pig serum *in vivo*(5), and evidence has been presented that they do not depend upon asparagine for growth *in vitro*(3).

In the present work, we have compared cells of the two lines for ability to incorporate L-valine-$C^{14}$ *in vitro,* in the absence of added asparagine and in the presence of asparagine in various concentrations.

*Materials and methods.* Lymphoma cells of each type were removed with aseptic technique from the peritoneal cavities of C3H mice of the Heston line procured from the Jackson Laboratory, Bar Harbor, 5 days following implantation of one million cells. The harvested cells were washed twice in Eagle's basal medium(6) in which phosphates were increased by 10 times and $CaCl_2$ had been omitted and which contained 5% horse serum. The concentration of cells was then adjusted to 2 million per ml with the aid of a Coulter electronic cell counter. One ml of the cell suspension was next added to 1 ml Eagle's basal medium to which certain additions had been made so that the final incubating medium contained: 5% horse serum, uniformly labeled L-valine-$C^{14}$ (Nuclear-Chicago Corp.) 0.1 $\mu$c, 24 $\mu$g per ml, and, in certain tubes, L-asparagine-$H_2O$ (Mann Research Lab.) in various concentrations.

Incubation was carried out during 4 hours at 37°C, following which the cells were removed from the suspension by centrifugation and washed once with cold Eagle's medium without serum. Cold 10% trichloroacetic acid (TCA) was next added; the cellular precipitate was washed again with 10% TCA, once with 100% ethanol, and finally dissolved in 2 ml hydroxide of Hyamine.† Counts were then made in a PPO-POPOP‡-toluene mixture with a Packard Tri Carb liquid scintillation spectrometer, each determination being made on triplicate or quadruplicate specimens. Counting efficiency was about 55%.

*Results.* Table I shows the results of two representative experiments in which RG1 and OG cells incorporated L-valine-$C^{14}$ in the

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† Hyamine = [p-(diisobutylcresoxethoxyethyl)-dimethylbenzylammonium chloride], Rohm and Haas.

‡ PPO = 2, 5-diphenyloxazole; POPOP = 1, 4-bis-2-(5-Phenyloxazolyl)-Benzene, (Nuclear-Chicago Corp.)