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Spontaneous Hemorrhage Induced by Administration of Aminoacetonitrile and Papain or Anticoagulants to Rats. (25162)

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The principal components of the matrix of epiphysial cartilage are collagen and chondroitin sulfuric acid-protein complex. Certain agents will damage, possibly selectively, each of these constituents. The lathyrogenic agent, aminoacetonitrile (AAN), appears to interfere with polymerization of tropocollagen molecules to collagen fibrils(1); hence, although total collagen content of cartilage from AAN-treated animals is not reduced, the physical properties of such tissue are greatly altered. Intravenous administration of crude papain leads to liberation of chondroitin sulfate from certain tissues, including cartilage (2), possibly as a result of enzymatic destruction of the protein to which the polysaccharide is bound(3). It was thought that the simultaneous effects of the 2 agents, AAN and papain, on cartilage, particularly reparative sequences, might be of interest. During the course of these studies, to be reported elsewhere, widespread, spontaneous subperiosteal hemorrhage has been observed. An exploration of the pathogenesis of this disease state is the subject of this report.

Materials and methods. Albino rats, weighing 45 to 50 g, were used in all experiments. Aminoacetonitrile hydrosulfate, generously supplied by Dr. I. V. Ponseti, was administered by stomach tube in either 1 dose of 20 mg or 2 doses of 10 mg/day. Crude papain was made as a 5% solution in physiological saline and filtered. The usual dose was .5 cc injected into femoral vein. Heparin sodium was administered intravenously, .5 mg being the usual amount. Sodium polyglucose sulfate, generously supplied by Dr. Peter Mora, was administered intravenously, 5 mg being the usual dose. Dicoumarol was added to ground laboratory chow to make a concentration of .1%. Care was taken to cauterize the site of the intravenous injection of papain or anticoagulants lest the animal die of hemorrhage. At autopsy blocks of tissue were fixed in neutral formalin and processed in standard fashion.

Observations. When AAN is administered to normal rats, profound effects are found in rapidly growing epiphysial cartilages in 24 to 48 hours(1). At this time the periosteum may easily be detached from the tibia or calvarium. Gross hemorrhage in these areas is not common at this time, nor is bleeding conspicuous about the ribs, though microscopic subperiosteal hemorrhages are often seen after 2 to 3 days' administration of AAN.

If crude papain is administered intravenously to animals which have received AAN for 1 or 2 days, the rats become listless after 1 to 2 hours. The coat becomes ruffled; in a

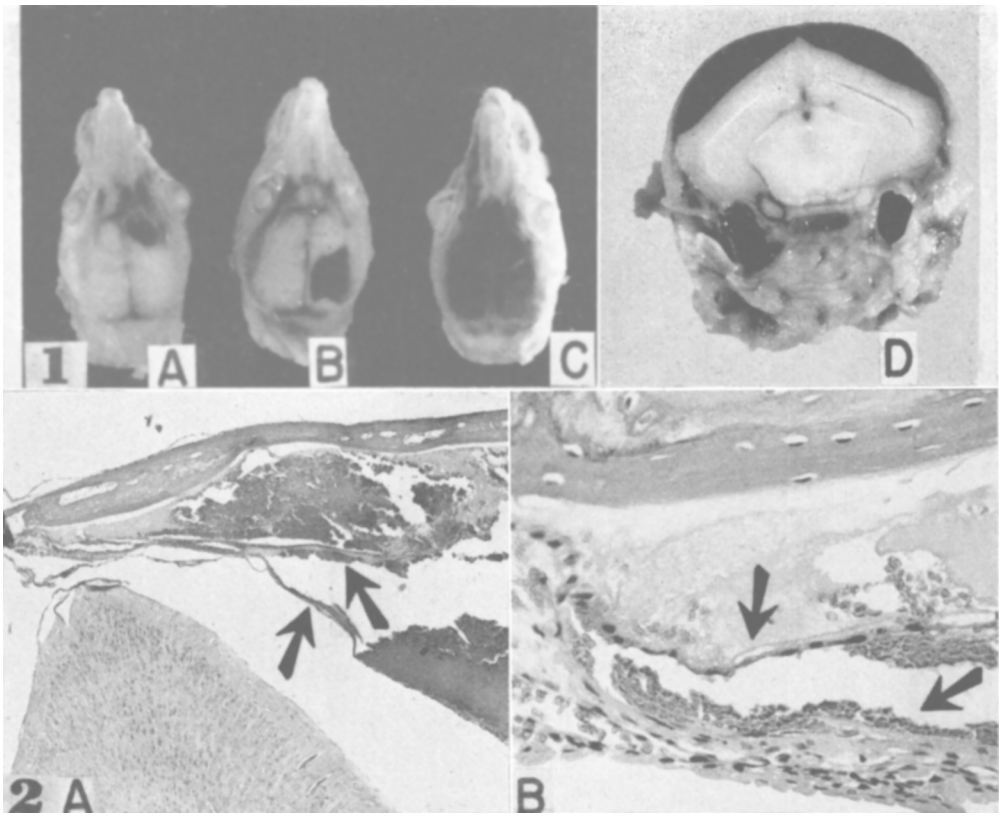

FIG. 1. Representative skulls of rats receiving 20 mg AAN 36 hr before intravenous injection of papain. Animals *A* and *B* were sacrificed at 8 hr; *C* died in 6 hr. *D*. Cross-section of head through parietal bones to show bilateral extradural hemorrhage and brain compression. This animal died 6 hr after papain administration.

FIG. 2 *A*. Low power (× 35) cross-section through parietal bone and underlying brain. Hemorrhage is present between 2 distinct membranes (arrows) which are shown more clearly in *B* (× 300). Here from above downward are shown: bone, periosteal cell layer (arrow) separated from it, and dura (arrow) separated in turn from the periosteal layer.

short time some animals are moribund. The earliest lesion to appear is discoloration over the vertex of the skull. When hair is removed, the discoloration is plainer and seems to be associated with the skull itself. When an animal showing this change is anesthetized and the skin is reflected, widespread hemorrhage is found beneath the cranial bones. The hemorrhage is usually, though not always, bilateral and may extend into the snout region (Fig. 1). The orbit may be involved so that exophthalmos, sometimes bilateral, is seen. Such rats usually die in 6 to 8 hours. Some, as noted below, live. During life hemorrhages have not been noted elsewhere.

At autopsy the extent and effects of hemorrhage beneath the skull are confirmed, and can be determined (Fig. 1D). Moreover, hemorrhage is found in several other characteristic sites: beneath the periosteum of inner surfaces of ribs or about the tibiae and scapulae, and in the orbital areas. Virtually no hemorrhage is found in the viscera. On microscopic examination the exact site of the intracranial hemorrhage can be delineated (Fig. 2). The periosteum, which consists of a single layer of cells, is cleanly separated from the overlying bone by blood cells. In addition, the single layer of periosteal osteoblasts may be found to have been dissected away from the more fibrous dural layer by red blood cells. As a result, 2 distinct membranes lie between the cranial bones and arachnoid-covered brain. Subdural hemorrhage is not

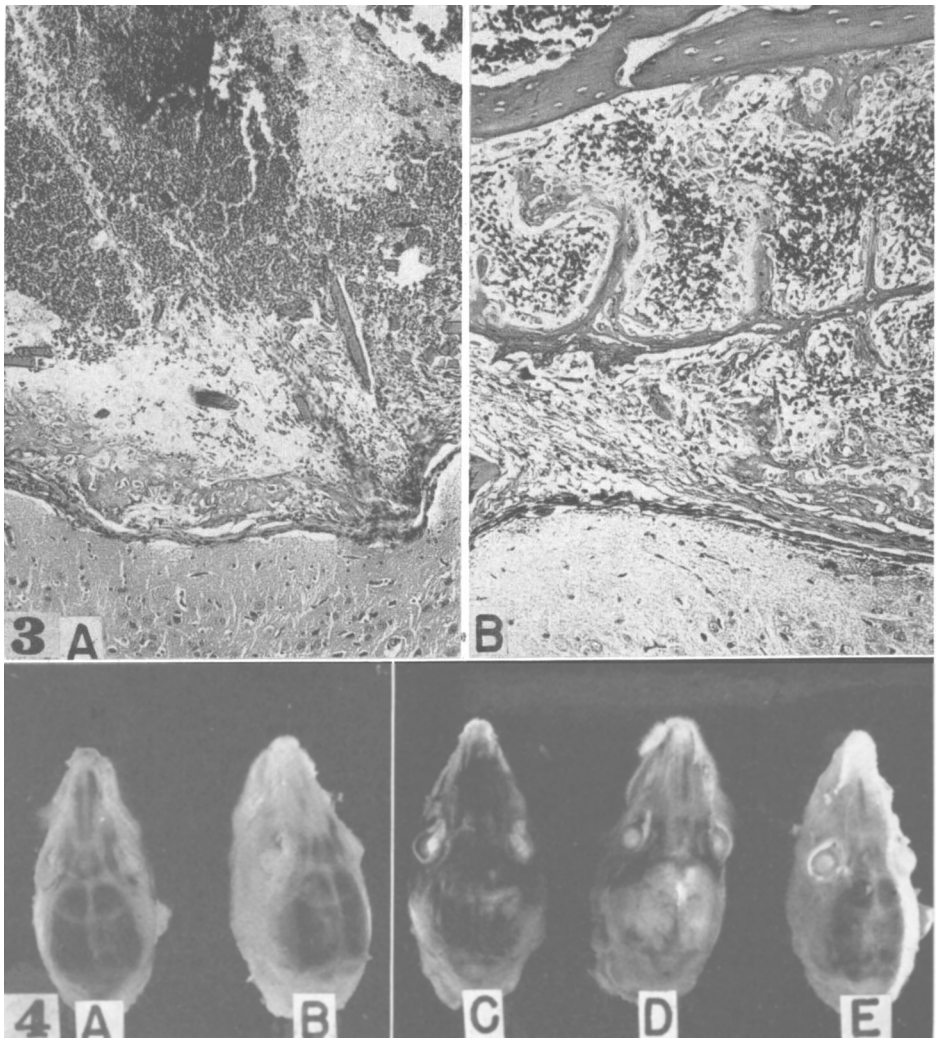

FIG. 3. *A.* Periosteal layer and dura which have been dissected from bone by hemorrhage and are compressing underlying brain (× 100). Lesion is 3 days old. Note bone, including cartilage, which has formed in this time. *B.* Similar area (× 85) from animal in which hemorrhage occurred 5 days previously. Bone formation here is more marked.

FIG. 4. External appearance of skulls of rats which had received 20 mg AAN 24 hr before administration of: *A,* heparin and *B,* polyglucose sulfate. Skulls *C, D,* and *E,* are from animals on dicoumarol diet for 36 hr to which AAN had been administered 24 hr before.

present. No hemorrhage is found beneath the external periosteum of the cranium. However, sections through the anterior portion of the skull reveal that hemorrhage is present beneath the periosteum on the outer surface of orbital bone, thus explaining the exophthalmos seen during life. Sections of ribs and long bones reveal separation of the periosteum from their cortices by red blood cells.

If animals do not die of the immediate effects of hemorrhage, the course of organization of the sub-periosteal or extra-dural intracranial hematomata may be followed. Such observations do not help in understanding the pathogenesis of the lesion, but are of interest in that they provide a method for study of new bone formation. The periosteal cells immediately begin to proliferate to form osteoid; this then calcifies, becoming true bone. Moreover, in some cases these same cells appear to

give rise to cartilage which is then replaced by bone (Fig. 3).

When papain is administered in similar doses to animals which have not received preliminary treatment with AAN, no hemorrhage occurs. Larger doses, however, may lead to bleeding, particularly between myocardial fibers. Subperiosteal hemorrhage is not seen. Intravenous administration of papain has been recognized for some time to inhibit blood coagulation(4). The anti-coagulant activity of papain is not clear, though it has been suggested that a heparin-like substance is liberated(5). It seemed possible, therefore, that the hemorrhage produced by papain in animals pretreated with AAN may have been due to the anti-coagulant activity of some material liberated by the enzyme. Consequently, the effects of other anticoagulants have been investigated.

When heparin or polyglucose sulfate, which has a similar anti-coagulant activity(6), are administered to rats which have been treated for one or 2 days with AAN, a syndrome develops which is entirely similar to that just described (Fig. 4). The behavior of rats during life and the gross and microscopic findings at autopsy are identical to those which have been observed following administration of papain. At dose levels administered neither heparin nor polyglucose sulfate leads to spontaneous hemorrhage; however, with larger doses generalized bleeding may be encountered.

Finally, the effect of lowering certain of the essential constituents of the coagulation mechanism has been investigated. Animals were fed dicoumarol in the diet; AAN was administered at varying times thereafter. The results here are complicated by the toxic effects of the drug on growth of rats. If no growth occurs and hence no new matrix is produced, the effects of AAN will be negligible. Hence, the results are not as clear-cut as those produced by papain or the anti-coagulants which have an immediate effect. However, intracranial as well as sub-periosteal hemorrhages have been observed in 8 of a series of 10 animals treated with AAN after being on the dicoumarol-containing diet for 36 hours (Fig. 4).

Discussion. Hemostasis is usually thought to be dependent on the integrity of capillary walls, as well as conditions within and without the lumens of these structures. Normal blood coagulation mechanisms, including platelets, are of importance within vascular lumens. Externally the support of small vessels by connective tissue fibers must be of great importance. The pathogenesis of the hemorrhage encountered can only be speculated upon, since the precise damage to the capillary wall and pericapillary structures must await study with the electron microscope.

An explanation for the lesions which develop as a result of feeding sweet pea seed or chemical agents, such as AAN, has been developed as a result of studies of epiphysial cartilage(1). It is concluded that the defect results from failure of the tropocollagen molecule to polymerize into mature collagen fibrils. In those areas where, normally, new blood vessels are actively proliferating, constant supply of collagen fibers is needed for their support. During the period of AAN administration collagen fibrils presumably are not formed, hence, as capillaries grow they are devoid of their normal collagenous supporting structures. The undersurface of the cranial vault and the subperiosteal areas of long bones and ribs are sites where blood vessels are actively proliferating, perhaps more so than at any other place in the body. Hence, these areas will show the effects most prominently.

When hemorrhages were first encountered in AAN-treated rats following papain administration, we naturally thought that the now well-recognized effect of this enzyme on polysaccharide-protein complexes was likely to be a factor(2). Hence, an alteration of some polysaccharide-protein complex in or about blood vessels with consequent interference with their integrity was considered. Such may be the case. However, we were also aware of the *in vivo* anti-coagulant activity of papain as reported by Kellner, *et al.*(4); moreover, Monkhouse(5) had suggested that heparin-like substances might be liberated by the enzyme. Since papain removes compounds such as chondroitin sulfate A and C from the tissues(2), might not other sulfated polysac-

charides which, in contrast, have anti-coagulant activity, such as chondroitin sulfuric acid B(7) be liberated as well? Such reasoning led us to study the effects of heparin and polyglucose sulfate, in addition to the well-known action of dicoumarol on the coagulation mechanism. The results obtained with these materials clearly involve the blood clotting mechanism, for each produced hemorrhage in AAN-treated animals but by themselves did not. Little would be gained by discussing the mode of action of these materials. The problem is far too complex; in order to elucidate it completely each of the many blood clotting factors which are altered by these agents would have to be investigated. It seems necessary to add that in addition to coagulation factors *per se*, on the clotting mechanism, the role of heparin or dicoumarol on the integrity of the vascular walls themselves must also be kept in mind.

Another outcome of these experiments is of interest to the student of osteogenesis. For as already described, the hemorrhage dissects away the periosteal layer of osteoblasts and hence, affords a means of studying new bone formation by these cells when the effects of the lathyrogenic agent and accessory factors such as heparin or papain have worn off.

Summary. When appropriate amounts of the lathyrogenic agent, AAN, are administered together with papain or the anti-coagulants, heparin or polyglucose sulfate, widespread spontaneous subperiosteal hemorrhages are seen. These do not occur when these materials are administered by themselves. The possible underlying mechanisms of this change are discussed.

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Tobacco Mosaic Virus Reconstitution Using Inactivated Nucleic Acid.* (25163)

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The conditions required for inactivation of Tobacco Mosaic Virus ribonucleic acid (TMV-RNA) by formaldehyde, nitrous acid and other reagents have been described(1-5). It was clearly shown that in each case the rate of inactivation is considerably greater for the RNA than for the intact virus, and with several reagents first order kinetics of inactivations were observed for the RNA, while the virus appeared to become progressively more resistant. The question then arose whether inactivated RNA could combine with native TMV protein to give stable virus rods. For, such reconstituted inactive virus might be expected to represent a perfect antigen for purposes of immunization against the virus.‡ In contrast to the chemically inactivated virus, the protein of which is of

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‡ The viral protein alone, free from nucleic acid, might be assumed to represent the ideal agent for immunization. However, Aach(6) has recently shown that the dissociated TMV protein differs serologically from intact TMV, each having an antigenic component not shared by the other. Reaggregated protein rods reacted serologically like TMV, but these are not stable under physiological condi-