Hypervitaminosis A. An Adjunct to Present Methods of Vitamin A Identification.* (18937)

S. Burt Wolbach and Charlotte L. Maddock.

From the Division of Nutritional Research, The Children's Hospital, Boston, Mass.

The synthesis of vit. A and closely related compounds constitutes a field of investigation engaged in by numerous organic chemists. Once a new product has been prepared, its chemical structure elucidated and its spectrophotometric characteristics established, there remains the question of its biologic potency, for which no rapid methods of determination are available. The biologic assay procedure as given by USP XIV, including a depletion and assay period, requires a minimum of 46 days or a maximum of 73 days for completion. (U.S. Pharmacopoea XIV, p. 790, 1950).

This report offers a method for identifying as well as roughly evaluating the degree of biologic potency in terms of vit. A activity which is—(1) more rapid; (2) more specific; (3) requires no preliminary depletion period or use of specialized diets. This method depends upon the response of the skeletal structures of the young growing animal to excessive intake of vit. A. This response is specific in the sense that no other vitamin or any compound not closely related structurally to vitamin A gives a similar picture. The syndrome of hypervitaminosis A, with its emphasis upon changes in skeletal structures, has been repeatedly reaffirmed ever since its recognition by Collazo and his associates. Its characterization as an acceleration of normal growth sequences in long bones, first suggested by Wolbach and Bessey(1) was fully described by Wolbach(2). This response is readily recognized histologically in suitably prepared material when high dosage levels are maintained even for short periods of time. If one uses a weanling rat (the test animal that is most convenient and responds with great uniformity at high dosage levels) a tentative diagnosis can be made within 5-6 days, to be confirmed by histological findings in another 10 days, if the material under investigation has a potency in the range of vitamin A. If its potency is less, the experiment may consume a period of 3-4 weeks. If no potency is demonstrable on the basis of the histological picture at that time one can, with safety, state that the product compares so unfavorably with vitamin A that its potency is negligible.

The method has limitations in that it can be used only: (1) where high concentrations of a substance are available, and (2) where histological technics for bone study purposes are available and the interpretation can be made by one familiar with normal growth of rat bone.

Method. Weanling rats (their breed is immaterial) 21 days of age, preferably 35-45 g in weight, are maintained on an adequate stock diet and fed amounts of the preparation at dosage levels which are the equivalent of .3 mg of vit. A alcohol per gram of body weight per day for a period of 5 to 6 days or longer, or until fractures are grossly evident. If there is enough of the preparation available, 3 rats should be selected, one to kill at the end of 5 or 6 days, or before if fractures are evident, one to continue for 14 days on this same regime, and one for 3 weeks. With potent preparations, animals will probably not survive the longer periods. At the same time a minimum of 2 animals of the same weight and age should be maintained on a dosage of .3 mg per g of body weight per day of vit. A in a preparation known to produce fractures within 5 to 6 days at this level. These should be killed when fractures become evident and will serve as control material against which the response of the product that is being tested may be compared. Initial weights of animals should be recorded, as well

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2. Wolbach, S. B., *Proc. Inst. of Med., Chicago,* 1946, v16, 4; and *J. Bone and Joint Surg.*, 1947, v29, 171.

as daily changes in weight. Daily food intakes should also be charted.

If the preparation compares favorably in potency with vit. A, the syndrome begins to manifest itself within 24 hours by loss of activity on the part of the rat, decreased food intake, failure to gain normally or to gain at all, and usually a slight reddish discharge around the eyes. These signs should be noted, also any tenderness on pressure over the long bones. Within 5 to 6 days the animal will show gait difficulties, favoring either one or the other hind leg, or a front paw. With further dosing, fractures will occur and may involve any or all long bones. The most common early site of fracture in our experience has been the tibia, the next most common the femur. With vit. A—and this applies to both the naturally occurring product and the synthetic vit. A—recognizable histologic changes will occur after 2 to 3 doses at this level of intake.

If the supply of material is limited and only one animal can be used, and if there are no evidences of fractures at the end of the 5 to 6 day period, it is advisable to carry the one animal through the 3-week period before killing. If the preparation compares at all favorably with vit. A, it will show results after this interval.

Animals when sacrificed are carefully examined, special attention being paid to the long bones and the structures surrounding them. Hemorrhages into muscles and connective tissue structures are usually evident around fracture sites. The bones, after 18-24 hours' fixation in Zenkers, with subsequent washing, are decalcified in a 5% aqueous solution of nitric acid, embedded in celloidin, and stained with hematoxylin-eosin.

The changes to be looked for as results of vit. A activity have been described(2). Preferred for this purpose are longitudinal sections through the knee joint, cut in the sagittal plane, including one-half to two-thirds of the length of tibia and femur. The changes produced by a potent preparation of vit. A are wholly the result of acceleration of normal growth sequences and include the effects of an increased rate of maturation and vascular penetration of epiphyseal cartilage cells and increased activities involved in remodelling processes of bone, including resorption of bone attended by increased numbers of osteoclasts and new deposition of bone in conformity with the normal growth pattern.

In a group of rats at any age before completion of growth there may be considerable variation in thickness of the cartilage of the proximal tibia and distal femur epiphyses. In hypervitaminosis A the epiphyseal cartilage in growing rats becomes thinner than the extreme of the normal variation. The reduction involves all layers of the epiphyseal cartilage because all sequences, with the exception of proliferation, are accelerated. If the account of the normal rat epiphyseal cartilage, as described by Dodds and Cameron(3) is followed, the zone of "reserve cells", their Zone 1 (the layer nearest the epiphysis) remains unchanged. We have seen no indication that this layer gives rise to new cells to be incorporated into Zone 2, the zone of flattened cells and cell multiplication. Zones 2, 3 (the zone of cell growth) and 4 (the zone of fully grown cells, with adjacent calcified cartilage matrix) all became much narrowed as a result of rapid growth and maturation of the cells composing these layers (Fig. 1). In young guinea pigs administered excessive amounts of vit. A, these sequences result in complete closure of the epiphyses of the long bones in a period of 10 to 12 days.

Knowledge of the normal is required for the appraisal of epiphyseal cartilage changes in the rat resulting from hypervitaminosis A, inasmuch as complete consumption of the tibial proximal epiphyseal cartilage does not occur. It is to be recalled that normally in the rat the epiphyseal cartilages of the distal end of the femur and proximal end of the tibia persist usually throughout the life of the rat. Not infrequently in the 5- to 7-day period of excessive vit. A administration to rats there is little apparent diminution in width of the epiphyseal cartilage since increase in size (growth) of the cartilage cells has resulted. The number of cells per column, however, is reduced at the expense of Zone 2, *i.e.*, the zone of flattened cells and cell multiplication.

3. Dodds, G. S., and Cameron, Hazel C., *Am. J. Anat.*, 1934, v55, 135.

FIG. 1. Proximal end of femur, anterior aspect. Rat, six days' administration of natural vitamin A concentrate, 1000 I.U. per g wt. Note hemorrhage, the result of rapid remodelling. 80 diameters.

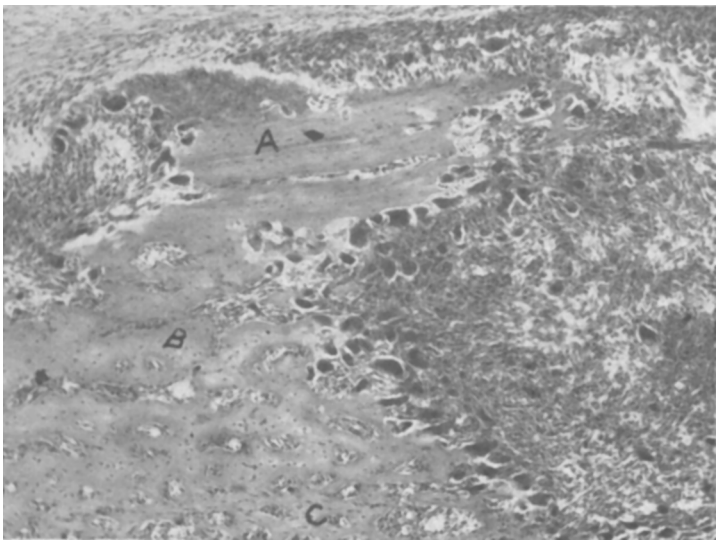

FIG. 2. Shaft of tibia. An oblique section through the tuberosity. Eight days' administration of synthetic vitamin A acetate. To show—(A) remnant of unresorbed bone; (B) region of new bone deposition. Note the extraordinary number of osteoclasts. 80 diameters.

The most marked reduction of width of the cartilage is at the periphery of the epiphyseal plate where there may be complete consumption of Zones 2, 3 and 4.

The changes in trabecular and cortical bone produced by excessive vit. A administration are so distinctive that less experience is required for their recognition.

The concavity of the shafts of bones—the tibia, for example—immediately adjacent to the epiphysis is maintained by resorption of the trabeculae as they merge into the cortical bone. In hypervitaminosis A a large excess of osteoclasts over the normal is apparent. The rapidity of resorption is such as to result in weakening of the periosteal attachments, hence small hemorrhages are often found in this region (Fig. 1). Farther

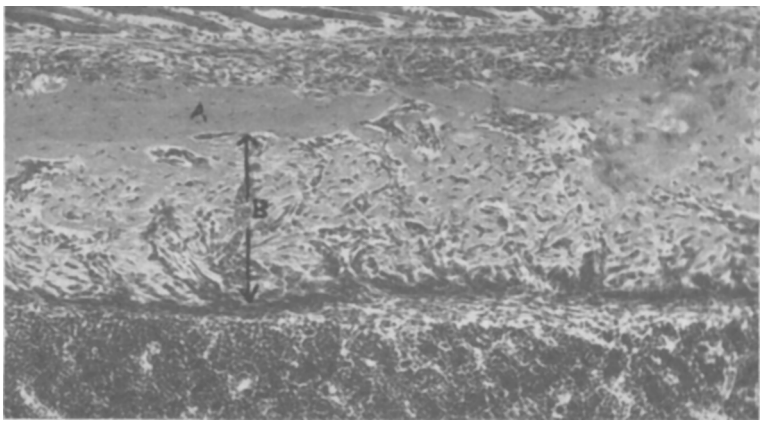

FIG. 3. Shaft of tibia, upper third. Three days' administration at 1000 I.U. per g wt, followed by 3 days at 1500 I.U. per g wt. Synthetic vitamin A acetate. Longitudinal section (A) and (B) to show the remains of the cortical bone still undergoing resorption; (C) new bone on the interior of the shaft. 113 diameters.

removed from the epiphyses where compact cortical bone must be removed, there is extensive osteoclasis on the external surface of the shaft and voluminous deposition of new bone on the interior of the shaft in the same regions (Fig. 2 and 3). This resorption and new deposition of bone follows precisely the normal growth pattern; only the rate of the processes has been changed. This has been determined by comparisons of normal femurs and tibio-fibular complexes with the corresponding hypervitaminosis A bones in serial sections.

If a compound compares favorably with vit. A in potency, fractures at the dosage levels used may occur in 5 or 6 days. They occur at sites where remodelling in normal growth is most active in regions subject to mechanical stress. Loss of previously formed cortical bone and lack of firmness of the newly formed bone explains the fractures. The lack of firmness of the newly formed bone is the result of incomplete calcification, expressive of the lag in calcification of bone matrix in normal bone growth as illustrated by the noncalcified matrix or osteoid present in appositional bone formation.

In applying this method for identification, the duration of the test period in the absence of fractures should be extended to 10 to 20 days, by which time the histological study of the bones should give the answer.

The advantages of the procedure may be summarized as follows:

(1) It is specific in the sense that no other substance administered under these conditions gives the response elicited by vit. A in skeletal structures. (2) No preliminary depletion period is necessary. This greatly shortens the time required for testing. (3) No special diets are required. (4) Numbers of animals in any one group may be very small—in fact, one animal is enough for a definitive answer. (5) Differences in potencies of vit. A preparations can be brought out by this method. For example, equivalent dosages of a synthetic crystalline vit. A acetate and of a concentrate of natural vit. A produced identical results with fractures by the sixth day.

Vit. A methyl ether in equivalent doses proved less potent than vit. A acetate or vit. A alcohol. There were no fractures during a 6-day period either with the crystalline product or with a concentrated solution supplied us by Dr. E. L. Sevringhaus, Director of Clinical Research of Hoffmann-LaRoche, Inc.† Results, *i.e.*, fractures comparable to those produced by the crystalline synthetic vit. A acetate or the concentrate of the natural vit. A were produced in one rat after

† We are indebted to Hoffmann-LaRoche, Inc., for the synthetic vit. A products used in these experiments.

23 days of administration. Four rats sacrificed after a 6-day period showed easily recognized histologic changes typical of excessive vit. A intake.

Vit. A phenyl ether proved to be without effect in rats run for periods of 6 to 11 days.

The limitations of the method are as follows: (1) It can be applied only when large dosages are used. The syndrome is not produced by small amounts of pure products or by oils low in potency. (2) In the absence of fractures, adequate histologic sections of bony structures (knee joint) must be used for diagnostic purposes. (3) The histology must be interpreted by one familiar with the typical response.

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Isolation of an Androgenic Compound from the Adrenal Venous Blood of Cows.* (18938)

FRANK X. GASSNER, DON H. NELSON,† HANS REICH, RICHARD T. RAPALA, AND LEO T. SAMUELS.

From the Department of Biochemistry, University of Utah College of Medicine, Research Laboratories Armour and Co., and the Colorado Agricultural Experiment Station, Fort Collins, Colo.

Although it has been thought that the adrenal gland may be capable of secreting androgenic compounds, the evidence presented has been inconclusive and other alternatives have seemed equally possible. Sayers(1) has suggested that such androgenic material may be the result of subsequent metabolism of the C_{21} steroids secreted by the adrenal cortex.

Besides 17-hydroxycorticosterone and corticosterone the presence of small quantities of other compounds in the adrenal effluent was indicated by previous work(2,3). The nature of these substances, however, was not determined.

In an attempt to obtain larger quantities of these compounds for identification, a special method was devised for the cannulation of the adrenal sinus of the cow. This has permitted the collection of large volumes of adrenal venous blood from cows stimulated with ACTH. At least 2 other compounds have been separated in addition to 17-hydroxycorticosterone(4). One of these fractions has now been demonstrated to have androgenic activity.

Experimental. Twenty liters of adrenal venous blood were collected from the adrenal sinuses of 3 cows which received an average of 600 mg ACTH intravenously during the blood collection. The animals also received intravenous infusions of heparin prior to the actual collection of blood and 5% glucose in saline during the 2-4 hour experiments. The blood was laked with an equal volume of H_2O and extracted with chloroform. The chloroform-extracted lipids were first partitioned between 70% ethanol and hexane and then between 30% methanol and hexane. The ketonic fraction of the 30% methanol-soluble lipids was acetylated and chromatographed on neutral aluminum oxide (Harshaw). Elution from the column was carried out with hexane-benzene, benzene, benzene-ether, ether-chloroform, and chloroform.

A fraction was eluted by the benzene-ether mixtures. This had an absorption maximum in the ultraviolet at 240 mμ and gave a positive Zimmerman reaction with maximum ab-

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† Public Health Service postdoctoral fellow, 1950-51.

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