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Effect of Toluidine Blue on Metastatic Calcification.* (20334)

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Hyperparathyroidism is the most common cause of metastatic calcification. Calcified lesions due to hyperparathyroidism have been reported in the connective tissue of the stomach, pulmonary alveoli and kidney(1). Within the kidney the peritubular basement membrane is involved. Gersh(2) considers the basement membrane as a plastic, well-organized ground substance that is highly labile in certain physiologic and pathologic states. Heller-Steinberg(3) and also Engel (4) have demonstrated that the histological changes seen in bone after administration of parathyroid extract, can be explained on the basis of depolymerization of connective tissue ground substance. Gersh and Catchpole(5) believe that calcification is most likely to occur in depolymerized matrix. Rubin and Howard(6) have confirmed this work and also observed that there is a relationship between the state of the connective tissue and the ability of the tissue to become calcified. They contend that more reactive groups are available to bind with calcium when the matrix is being depolymerized. The basic dye toluidine blue is known to combine with mucopolysaccharides of the ground substance. Sylven(7) and others believe this is due to the presence of chondroitin sulfate. Toluidine blue binds on reactive groups of the ground substance coinciding with calcified portions of the matrix. Furthermore, in three week-old rats in which calcification has not yet appeared, toluidine blue binds intensely with the matrix in precisely the geographical locations eventually to become calcified. Miller, *et al.*(8) have shown that *in vitro* calcification of rachitic rat cartilage is blocked by toluidine blue. These authors have demonstrated that this inhibition of calcification is directly proportional to the amount of toluidine blue present in the system. Marc and Wenk(9) and France(10) have reported that crystallization of certain inorganic salts from a supersaturated solution are markedly inhibited by dyes which stain the mother crystal.

Based on the above data, the object of this paper is to experimentally study the *in vivo* effect of dye binding on metastatic calcification produced in rats by the administration of parathyroid extract.

Methods. Young rats of the Sprague-Dawley strain, weighing 200-250 g and of the same sex, were used in the experiment. They were maintained on a routine laboratory diet and given water *ad libitum*. Fifteen rats each received a total dose of 1625 units of parathyroid extract (Lilly) administered over a 2 week period as described by Chown(11). Five rats each received 1625 units of parathyroid extract and 3 cc (intraperitoneally) of a 0.9 % saline solution each injection day of the experiment. Another group of 15 rats received the same dosage of parathyroid extract and in addition an intraperitoneal injection of 3 cc of a 0.04% solution of toluidine blue each injection day of the 2 week period. At the end of this time all animals were sacrificed by a blow to the head. Blocks of tissue obtained from the kidney, stomach, spleen, blood vessels and heart of each animal were fixed in absolute alcohol. Von Kossa and hematoxylin and eosin stains were obtained. To quantitate the extent of calcification in the

* This study was aided by Fred and Harvey Goldberg.

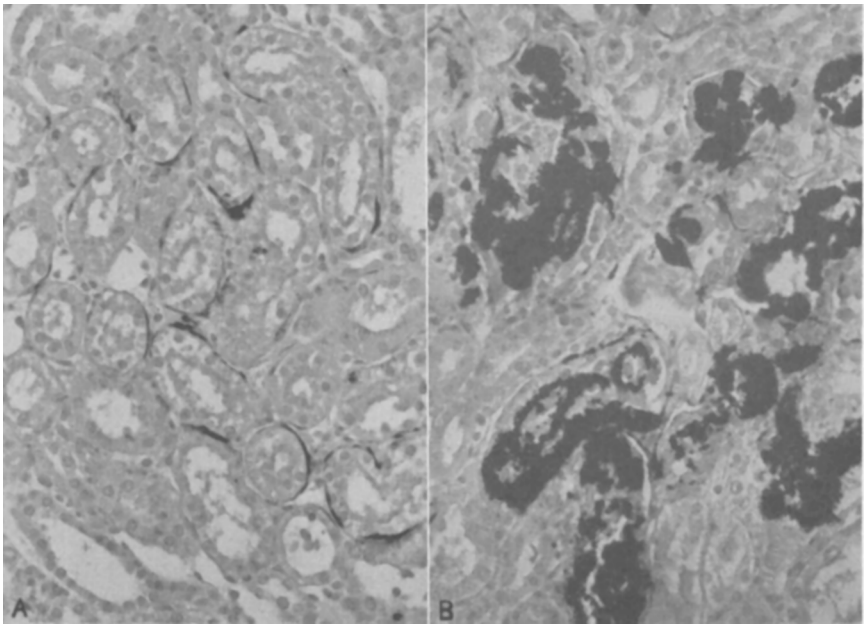

FIG. 1. A. Calcification in the basement membrane of the renal tubules in the rat produced by 1625 units of parathormone. (230 ×.)

FIG. 1. B. Augmentation of parathormone effect (1625 units) by concurrent administration of toluidine blue. (230 ×.)

kidney, four degrees of scoring were chosen. Those sections classified as 0 represent the stage at which no calcification, or at the most only small flecks of calcium, are seen in a few peritubular basement membranes. One plus refers to appreciable calcification, that is still localized peritubularly. When the calcium deposition appears in the basement membrane and also in the lumen of the tubules, it is termed 2 +; and 3 + refers to the situation in which the calcification is so extensive throughout the kidney that it is impossible to determine its exact location.

Results. Control animals generally demonstrated a uniform degree of metastatic calcification of the connective tissue ground substance in response to the 1625 units of parathyroid extract given. Calcification was observed in the kidney, blood vessels, spleen, heart and stomach. The administration of toluidine blue to animals receiving this same amount of parathormone greatly augmented metastatic calcification as compared to control animals given only parathormone. Figure 1 demonstrates the marked exaggeration of the parathyroid effect in the kidney produced by use of this basic dye. This augmentation of calcification was not confined to the kidney, but was observed also in the connective tissue matrix of the large and small arteries, spleen, heart and stomach (Fig. 2). The degree of calcification in the kidneys of the two groups of animals is presented in Table I. There was generally no calcification of the connective tissue ground substance in rats receiving a total two week dosage of 800 to 900 units of parathormone. The administration of toluidine blue in conjunction with 800 to 900 units parathormone, however, routinely resulted in calcification of 0 to 2 + in the ground substance of the organs and blood vessels previously described. Toluidine blue given alone failed to produce any calcification.

Summary and conclusions. 1. This experiment indicates that not only does toluidine blue fail to inhibit the metastatic calcification produced by parathormone, but that the deposition of calcium is augmented by the concurrent administration of the dye. Increased calcification was observed in the connective tissue ground substance of the kidney, stomach, spleen, arteries and heart. This was

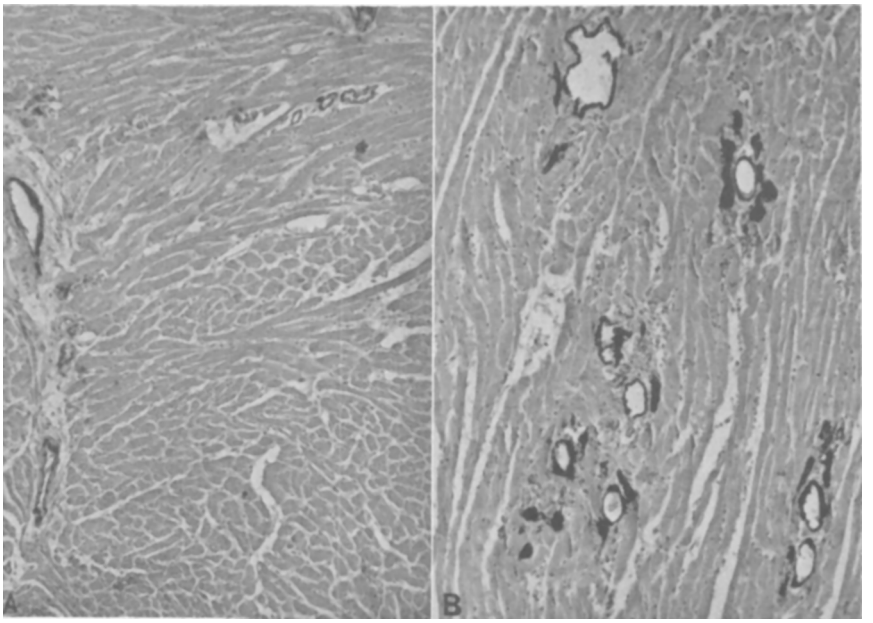

FIG. 2. A. Metastatic calcification of basement membrane of blood vessels produced by 1625 units of parathormone. (× 75.)

FIG. 2. B. Augmentation of parathormone effect (1625 units) by concurrent administration of toluidine blue. (× 75.)

an unexpected result due to the observation of Miller(8) that treatment of hypertrophic rachitic cartilage slices with basic dyes inhibits subsequent calcification. The fact remains, however, that the acid mucopolysaccharide present in the connective tissue matrix has a strong affinity for basic dyes. Rubin and Howard(6) have shown that the increased intensity of toluidine blue staining in areas about to calcify suggest that new polysaccharide appears or that the chemical state of the polysaccharide already present changes. An alteration in chemical form of the matrix favors combination with calcium and confers the state of calcifiability on the matrix. Engel (4) has reported that parathormone depolymerizes ground substance to increase calcifiability. It does not appear necessary therefore to infer competitive binding between toluidine blue and calcium for available reactive groups of the mucopolysaccharide. Concerning the data presented in this paper, toluidine blue apparently altered the chemical state of the ground substance, increased calcifiability, and exaggerated the parathormone effect. We have previously reported(12) that altering the connective tissue ground substance by compensatory renal hypertrophy or administration of desoxycorticosterone, increases or decreases, respectively, calcification produced by use of parathyroid extract. 2. The inhibitory effect of toluidine blue on the activity of heparin has been described by Allen(13). Experiments now in progress indicate that augmentation of calcification in the basement membrane of large and small arteries by use

TABLE I. Influence of Toluidine Blue on Metastatic Calcification.

No. of animals	Treatment	Duration, days	Degree of calcification in each exp. 0	+	++	+++
15	Parathormone (1625 units)	14	8	4	1	2
5	Parathormone (1625 units) and 0.9% saline	14	4	1	0	0
15	Parathormone (1625 units) and toluidine blue	14	0	0	5	10
8	Toluidine blue	14	8	0	0	0

of toluidine blue, as described in this paper, may be blocked by the concurrent administration of heparin.

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Nutritional Requirements of *Lactobacillus bifidus* Isolated from Poults and Chicks.* (20335)

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Romoser *et al.*(1) reported that large numbers of an unidentified branched gram positive organism were found in ceca of normal chicks. These organisms, later identified as *Lactobacillus bifidus*(2), were found in greatest numbers when 15% lactose was added to the basal diet and were decreased in number by the addition of 10 ppm of penicillin G. A high bifid count was correlated with poor chick growth. *L. bifidus* also constitutes 50% of the cecal population of turkeys(3). It is well known that under the usual growing conditions, poults grow faster when antibiotics are fed. This may be due to a reduction in numbers of bifid organisms. Since *L. bifidus* might utilize a known or unidentified substance(s) essential for rapid chick and poult growth, a knowledge of the nutritional requirements of avian bifids may contribute information concerning the mechanism of antibiotic action(4-6). Results of such a study are presented in this paper.

Experimental. Twenty-four cultures, tentatively classified as *L. bifidus,* have been isolated from the ceca of turkey poults. For the nutritional studies, 7 representative avian strains were selected, including 5 poult strains, one strain from adult turkeys(3) and one strain from chicks(2). The general procedures used in microbiological assays were employed for the determination of growth requirements. Stock cultures were transferred monthly on the basal medium containing 15 g per liter of Phytone (papaic digest of soybean, Baltimore Biological Laboratory) and 2% agar. The medium used for preparation of inoculum was of the same composition except for the omission of agar. Stock and inoculum cultures were incubated at 37°C, for 48 hours under anaerobic conditions using a Brewer anaerobic jar. The inoculum cultures were centrifuged, washed twice with physiological saline, resuspended and adjusted to a reading of 95 on the Evelyn photo-electric colorimeter, using

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