volving both nuclei and cytoplasm associated with inhibition of mitosis. These results are essentially in agreement with those described in this paper.

Our experiments are reported here in a preliminary fashion. As indicated in an earlier note(6) they are part of a project to investigate the influence of x-rays on the formation of chromosomal material. The accessory sex organs of the castrate rat seem to be a suitable object for studies of this kind as their growth response to TP is associated with rapid formation of desoxyribonucleic acid as indicated by tracer studies using P^{32}(8).

Summary. X-irradiation of the accessory sex organs of the castrate male rat and immediate subsequent treatment with testosterone propionate results in cellular hypertrophy with delayed onset of mitosis under the conditions of the experiment outlined above. If an interval of 5 days is allowed between irradiation and administration of testosterone propionate this delaying effect is lost.

We would like to thank Messrs. Edgar S. Greene, John G. Cohoe and Harley H. Nanney for their technical assistance.

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Ion Exchange and Recrystallization in Fixation of Ca^{45} in the Rabbit's Skeleton.*† (20984)

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Ion exchange has been demonstrated to be the principal mechanism for the fixation of radiocalcium and phosphate in bone, both *in vitro*(2,3) and *in vivo*(4-6). Calcium ions, in a solution of an ionized calcium salt, exchange with other calcium ions on the surface of bone crystals(3). Thus, under suitable conditions, it is possible to replace the calcium ions on the crystal surfaces with those of a calcium solution. In the studies herein reported Ca^{45} which remained on the crystal surfaces at various intervals after administration was determined by *in vitro* ion exchange removal in non-radioactive calcium solutions.

The purpose of these studies was to examine directly the role of ion exchange, crystallization and recrystallization in the fixation of calcium in newly formed and mature bone. No attempt will be made to review the concepts of surface chemistry of bone at this time. Recently an excellent review of the field has been made by Neuman(7). If all of a given amount of Ca^{45} were fixed in bone by ion exchange and remained on the crystal surfaces, then nearly all of the Ca^{45} should be removable by ion exchange with non-radioactive calcium solutions. On the other hand, if all of the Ca^{45} fixed in bone was incorporated into newly forming crystals, not more than 50% of the Ca^{45} should be removable by surface ion exchange.‡ The actual process of skeletal calcium fixation in a growing or mature ani-

* Preliminary results were presented at the Sixty-fourth Annual Meeting of Am. Physiol. Soc.(1).

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mal is a complicated combination of ion exchange and crystallization. In addition, there is the continuous process of recrystallization taking place in both newly formed and old bone(5,8,9). Formation of new crystals and recrystallization should reduce the *in vitro* exchangeability of *in vivo* fixed Ca^{45} in bone. These studies were in part designed to evaluate the rate and relative importance of these processes in calcium metabolism of bone. (Newman considers recrystallization as "a slow equilibrium of the crystal interior with the solution phase"(7).) After Ca^{45} is fixed on the surface of a formed bone crystal, recrystallization is the process which prevents its removal by *in vitro* ion exchange. These studies are, in part, comparable to those of Neuman using radiophosphate in rats(9). However, rabbits were used rather than rats because of their larger skeleton and Haversian system bone structure characteristic of large mammals. Further, Ca^{45} was used rather than P^{32} labeled phosphate because of its longer radioactive half-life and its greater specificity in relation to the inorganic phase of bone metabolism. Overall exchangeability of Ca^{45} was determined by applying beta-ray measurement technics to the bone sections before and after removal of the exchangeable Ca^{45}.

Method. Four 6 lb female, New Zealand white rabbits were given 0.5 mc/kg of Ca^{45} as the chloride (a total of 0.5 mg of calcium) intravenously. Animals were sacrificed at 1 hour, 24 hours, 1 week and 3 weeks following a single intravenous administration of Ca^{45}. The third and fourth lumbar vertebral bodies were longitudinally halved. One half of each vertebral body was fixed in acetone for 48 hours. The other halves were fixed in 4% formaldehyde solution containing ~1% sodium borate. Formalin fixed tissues were washed for 12 hours in 2 changes of distilled water prior to dehydration in alcohols. Dehydration of acetone-fixed tissue was completed in one change of acetone, prior to placing in 50:50 ether-alcohol. Five to 10 μ sections of the undecalcified bone embedded in plasticized nitrocellulose were prepared by a technic that we have previously reported (10). Sections were mounted on gelatinized glass slides and the embedding medium was then removed in absolute acetone. Sections were treated in .01 M CaAc§ containing .01 M boric acid and ~.0006 M Na ions. Control sections were treated with the buffer solution alone as well as distilled water. The results reported are on acetone-fixed bone sections except when formalin-fixed material is specified. Sections were treated with buffer solution prior to CaAc treatment as well as after CaAc treatment. All treatments were carried out under the following constant conditions: (I) Temperature, 18°C; (II) time, 1 hr; (III) solution volume, 50 ml; (IV) agitation, constant and slow; and (V) pH 7.7-7.8 (except for the distilled water whose pH was 6.2-6.5). Serial sections were so distributed that all comparative studies on a single animal were as representative as possible of the bone of the vertebral bodies. A group of 4 representative bone sections from an animal were treated simultaneously. Four groups of sections, one from each animal of the four sacrifice periods were treated at the same time and under the same conditions. Each study was repeated 3 or more times. Other groups of bone sections from the animals of the four sacrifice intervals were treated in buffered CaAc for periods varying from 2 minutes to 96 hours. Individual sections were counted in a windowless proportional counter before and after each treatment. The loss of activity‖ from individual sections was calculated and corrected for radioactive decay. All counting data was normalized to a Ca^{45} standard counted before and after each section. In order to evaluate the possibility of small microscopic fragments of bone being lost from the bone sections in the process of exposure to agitated solutions, 6 different

‡ *In vitro* studies indicate that the fraction of phosphate in a bone crystal which is present on the surface is less than 15% in old mature bone and 50% or greater in newly deposited bone(8). All evidence to date indicates that this relation would also exist for calcium ions qualitatively and probably quantitatively.

§ In the following text, for reasons of brevity, all solutions referred to as CaAc are .01 M calcium acetate containing the above stated borate buffer.

‖ Activity is the number of beta disintegrations detected per unit of time under the conditions of measurement.

TABLE I. Ca^{45} Desorption from Bone Sections by 1 Hr Treatments in CaAc and Buffer Solutions.

Sacrifice time following Ca^{45} admin.	% of Ca^{45} desorbed by treatment in: CaAc	Buffer
1 hr	56.20 ± .94	27.47 ±1.06
24 "	29.73 ±1.05	11.60 ± .96
7 days	16.60 ± .57	5.15 ± .74
21 "	15.10 ± .78	7.00 ±1.23

treatment solutions containing the large quantities of Ca^{45} leached from bone sections were filtered through fine fritted glass discs. In no case was any detectable activity present on the surface of the discs. These results would indicate that no appreciable loss of Ca^{45} occurred due to fragmentation of the bone under the experimental conditions. When fragmentation did occur it was always visably obvious and the results correspondingly unreasonable.

Results. When sections of bone from the different animals were treated in CaAc solution, the fractional loss of Ca^{45} content decreased with increasing sacrifice period. This loss was 56.2 ± .94%¶ from the bone of the rabbit sacrificed at 1 hour. Table I and the solid line of Fig. 1 show the decrease in the fraction of Ca^{45} leached** in CaAc as a function of time following Ca^{45} administration.†† Control treatment of bone sections in the buffer solution alone produced a loss of 27.47 ± 1.06% of the initial Ca^{45} from the bone of the 1 hr animal. The loss of Ca^{45} in buffer solution from the bones of the animals of the 4 sacrifice intervals are also listed in Table I. No statistically significant difference was observed in the data between Ca^{45} loss in buffer and distilled water. When groups of sections were treated first in buffer solution and then in CaAc, the total loss was found to be comparable to treatment in CaAc alone. The bone of the 1 hour animal regularly lost 25 ($s‡‡ = 2$)% and 33 ($s = 3$)% of the initial activity in the first and second treatments, respectively. When formalin-fixed bone sections from the same animal (which had been washed in distilled water during processing) were treated first in buffer solution followed by CaAc, the loss of activity was only 16 ($s = 2$)% in the buffer solution; however, the loss in CaAc solution was statistically the same as that which occurred in acetone-fixed sections.

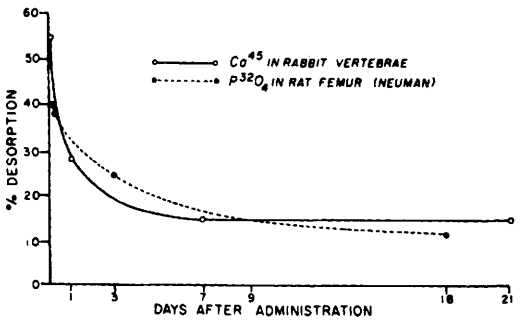

FIG. 1. Plot of % of Ca^{45} desorbed from bone section by 1 hr treatment in CaAc as a function of the time following administration, and comparison to radiophosphate data of Neuman(8).

These studies indicate that Ca^{45} is present in 3 or more forms in the acetone-fixed bone sections of the animals sacrificed at 1 and 24 hours after Ca^{45} administration. These forms are as follows: 1. That which is lost in distilled water or buffer solution, and 2. That which is exchangeable with calcium ions over and above the fraction lost in water or buffer, and 3. That which is not removable by *in vitro* ion exchange. The data indicates that the first 2 fractions are independent of one another, as demonstrated by the fact that removal of the first does not appreciably affect removal of the second. For convenience, the Ca^{45} fraction lost in distilled water or buffer will be referred to here as the loosely bound fraction, and that fraction which is removed only by ion exchange is referred to as the

¶ Plus or minus values given are standard errors of loss from individual samples in groups of bone sections.

**Leaching is a general term denoting loss by any mechanism.

†† When Ca^{45} is exchanged onto bone section *in vitro* in the course of 1 hour in radioactive $CaCl_2$ solution 77 ± 4% of it is removed in a similar treatment in non-radioactive $CaCl_2$(11). This would indicate that the treatment of CaAc under the conditions used removed approximately 3/4 of the Ca^{45} present in crystal surfaces. From this study it is apparent that the results reported in Table I as loss in CaAc treatment are slightly smaller than the true value of the total exchangeable Ca^{45} fraction.

‡‡ s = the standard deviation from the mean value of all evaluating experiments.

firmly bound fraction. From all studies, the loosely bound fraction constitutes 25 ($s = 2$)% and the firmly bound fraction 32 ($s = 3$)% of the initial Ca^{45} in the bone sections of the 1 hour animals. The loosely bound fractions represent a little less than ½ and the firmly bound fraction a little more than ½ of the Ca^{45} lost from the bone section by 1 hour CaAc treatment. This same relation exists for the relative magnitudes of these fractions in the bone of the 1 and 24 hour animals. The data are not sufficiently good for the 1 and 3 week animals to make a definite statement because of the increased error in measuring small differences. The results obtained on formalin-fixed bone sections parallel those obtained on acetone-fixed material. The exception to this being that the loosely bound fraction was slightly smaller in the formalin-fixed than acetone-fixed bone. This decrease in the loosely bound fraction of formalin-fixed bone may be due to its partial loss in the course of distilled water washing during pre-embedding processing. Rough calculations indicate that 10-20% of the Ca^{45} in the bone sections of the animal sacrificed after 1 hour should be present in the bone marrow, blood, etc. It is very likely that soft tissue Ca^{45} makes up a large component of the loosely bound Ca^{45} fraction in the 1 hour animal.

When bone sections were treated in CaAc for increasing periods of time, there was an initial rapid followed by a slow progressive loss of the Ca^{45} from the bone sections of the 4 sacrifice intervals (Fig. II).

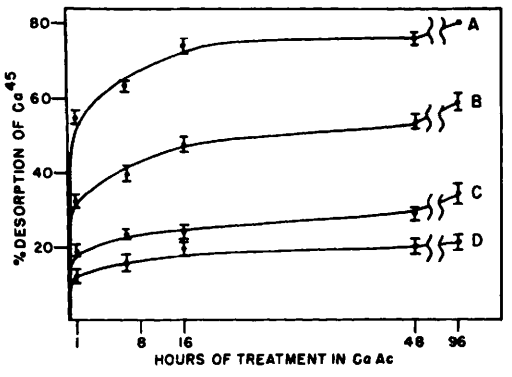

FIG. 2. Desorption of Ca^{45} from bone sections, as a function of time of treatment in CaAc. Sacrifice times of animals following Ca^{45} administration are: A. 1 hr, B. 24 hr, C. 1 wk, and D. 3 wk.

The shape of the curves of the rate of Ca^{45} loss may be explained by the following: (a) Ion exchange of crystal surface Ca^{45} with solution phase calcium is an exponential function with respect to time(3) and (b) *in vitro* recrystallization is probably occurring continually(8), uncovering previously buried Ca^{45}. It is thought that this process of recrystallization does not produce an appreciable renewal of crystal surfaces in most systems during the first 72 hours of exposure to a solution phase(9). There is unquestionably some overlap in the loss due to pure exchange of crystal surface Ca^{45} and that due to release of buried Ca^{45} by recrystallization. The time interval of 1 hour was arbitrarily chosen for purposes of experimental convenience, as well as obtaining results well situated on the plateau of loss. The difference in the fractional loss of Ca^{45} from bone sections of animals representing the four sacrifice intervals become increasingly apparent with increasing time of treatment in CaAc. Differences exist between the loss from the bone of the 1 and 3 week sacrificed animals after the 96 hour treatment, which was not as apparent after the 1 hour period. The interpretation of the 96 hour values for the four animals is difficult in view of the occurrence of *in vitro* recrystallization. These data indicate that *in vivo* fixed Ca^{45} progressively becomes more deeply incorporated in the lattices of bone crystals as time passes.

Discussion. Neuman demonstrated that radioactive phosphorus which was fixed *in vivo* in the skeletons of young and old rats was rapidly made unavailable to *in vitro* removal by ion exchange technic(9). The work herein reported demonstrates that Ca^{45} deposited *in vivo* in the slowly growing skeleton of rabbits is likewise made unavailable to removal by ion exchange. The similarity of results is demonstrated in Fig. 1 where both sets of data are shown.

From the reported data it is concluded that approximately 50% of the Ca^{45} present in the bone of the rabbit sacrificed 1 hour following intravenous Ca^{45} had been incorporated in a non-exchangeable form of bone

mineral. At 24 hours and 21 days following Ca^{45} administration this non-exchangeable fraction had increased to 70 and 85%, respectively. From these data, it may be concluded that a large fraction of the crystal surface calcium ions are constantly being converted to nonexchangeable intracrystalline positions in bone as a whole.

In separate investigations paralleling the reported experiments, the exchangeability of *in vivo* fixed Ca^{45} has been determined in separately studied areas of mature and forming bone by quantitative radioautographic technics(11,12). These studies show that deposited Ca^{45} becomes unavailable to *in vitro* ion exchange in mature bone at almost the same rate as that of newly formed bone. Thus the reported data for the change in availability of deposited Ca^{45} to ion exchange on bone as a whole are equally applicable to both areas of mature and forming bone (separately).

It is unmistakably apparent that the vast majority of the Ca^{45} ions in bone are initially fixed *in vivo* by the process of ion exchange (7). The *in vitro* exchangeable ions are usually considered to be those contained in the first layer or surfaces of bone crystals(7). The processes by which *in vivo* fixed Ca^{45} becomes unavailable to *in vitro* ion exchange removal (placed in intracrystalline positions) are (I) incorporation in newly forming crystals and (II) entrapment by the poorly understood process of recrystallization. The 2 processes are unquestionably occurring simultaneously in areas of forming bone, and probably only the latter is taking place in areas of mature bone(9). One may consider the rate at which the *in vivo* fixed Ca^{45} becomes unavailable to *in vitro* ion exchange removal as the rate of transformation of crystal surface ions to intracrystalline positions. When this is done with the reported data and that of Neuman(9), it is apparent that the half time of the crystal surface to intracrystalline transformation rate must be less than one hour. This is a surprisingly rapid rate for such a transformation to be taking place in the solid inorganic phase of bone where metabolic activity has frequently been considered to be relatively inactive.

The problem of dissolution versus exchange loss of Ca^{45} from bone sections treated in various manners must be considered. It was not possible in these studies to quantitatively measure the loss of calcium from treated bone sections.§§ Since approximately 20% of the Ca^{45} fixed on bone sections by *in vitro* exchange is invariably lost by treatment in distilled water(11), it is assumed that 20% of the crystal surface bound calcium is quite soluble. This extremely soluble fraction may represent calcium which is present in the recently described hydration layer of bone crystals(13). It is not unreasonable to assume that the loss of Ca^{45} during treatment of bone sections in buffer solution, in the reported studies, was substantially due to simple dissolving of calcium from crystal surfaces. This type of loss should be considerably smaller in CaAc treatment since the solubility of hydroxypatite is dependent upon the calcium and phosphate ion produced (among other things(7)). This problem will be considered more in detail in separately reported studies (11).

Summary. Undecalcified bone sections of rabbits sacrificed at intervals between one hour and 3 weeks following Ca^{45} administration were treated in calcium acetate solutions. This procedure was designed to determine what portion of Ca^{45} remains on crystal surface of bone as a function of time following administration. The data are interpreted as indicating that recrystallization and crystallization processes are removing calcium to non-exchangeable positions at extremely rapid rates. At one hour following Ca^{45} administration, ½ of the isotope fixed in bone is no longer available to removal by ion exchange technic.

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§§ Analyses done on bone section and treatment solutions reveal that less than 10% (.01 mg) of the total calcium of the bone was lost in buffer treatment.

of the authors indebted to Dr. C. P. Leblond of McGill University for his stimulating influence in this field of endeavor.

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Effect of Propylene Glycol on Methemoglobin-Forming Capacity of p-Chloroacetanilide in Rat and Guinea Pig.* (20985)

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Propylene glycol has been used extensively as a non-toxic and metabolically inert solvent for the oral or parenteral administration of various water-insoluble compounds to animals and man. Studies concerning gastric secretion in rats(1), however, have shown rapid secretion by the stomach of Toluidine Blue when injected in aqueous solution, but no secretion when injected in propylene glycol solution. The supposedly innocuous nature of propylene glycol has also been disputed: a marked hypertensive effect in cats, rats, and rabbits(2), and LD_{50} doses in rats of 13 ml/kilo intraperitoneally and 28 ml/kilo orally(3) have been reported. In view of these findings, the effect of p-chloroacetanilide on the blood of rats and guinea pigs in presence and absence of propylene glycol was investigated.

Methods. Adult male Wistar rats, 200-400 g, and adult female guinea pigs, 500-630 g, were used. All animals received tap water and food *ad libitum*. The rats were maintained on Purina Laboratory Chow pellets and the guinea pigs on Purina Rabbit Laboratory Chow pellets. The test doses (Table I) were administered by intraperitoneal injection. p-Chloroacetanilide, only slightly soluble in water, was suspended in a finely divided state in 0.1% methylcellulose solution; the methylcellulose being used for its wetting property. Two hours after injection, 2 ml blood samples were obtained by heart puncture under light ether anesthesia. Heparinized syringes were used to prevent blood coagulation (3 mg sodium heparin/ml distilled water). Cell volume to plasma volume ratios were determined by the hematocrit method of Wintrobe and Lansberg(4); total blood pigment by the method of Sahli(5); and methemoglobin by the method of Michel and Harris(6).

Results. The results are summarized in Table I. A dual effect on the blood is evident. The injection of propylene glycol produced an increase in hematocrit over the normal value. There was a corresponding increase in total blood pigment which indicated loss of fluid from the blood. This increase in hematocrit was uniform in both species and was not influenced by the presence of p-chloroacetanilide. On the other hand, p-chloroacetanilide produced methemoglobinemia in both species in presence and absence of propylene glycol. The methemoglobin-producing effect of a propylene glycol solution of p-chloroacetanilide, was greater in the rat (8.09%) than in

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