TABLE IIa. Incubation of Supernates from Tissue Cultures.

Tissue	Medium tested before feeding	After 5 days of growth	Days of incubation of supernates alone at 37°C	# samples	Mc +	No mc
Rat subcut.	No Mc	Mc +	4 - 18	9	9	—
Chick embryo bone	"	"	8 - 33	41	38	3
Rat tail tendon	"	"	4 - 55	7	6	1
Rat subcut.	"	"	30*-195*	3	3	—
Chick embryo bone	"	"	6*- 65*	22	21	1

* Kept in refrigerator at 4°C.

time, usually as long as the cultures do not deteriorate, while replica cultures, for instance strain L, sooner or later lose the ability to produce hyaluronic acid, despite their excellent appearance and good growth. 3. Although it has not yet been possible to separate factors promoting mucopolysaccharide production from factors promoting growth, it has been observed that cultures kept at room temperatures with considerably reduced growth, may sometimes continue to produce hyaluronic acid. 4. Hyaluronic acid of chick embryo extract is depolymerized in the first few days of incubation at 38°C (Table II), while hyaluronic acid produced in tissue culture is not depolymerized for months under the same conditions (Table IIa). A positive mucin clot test after a few days of incubation thus always indicates the presence of hyaluronic acid newly produced *in vitro* by fibroblasts. Chick embryo extract may therefore be used in any concentration in the culture medium in which production of hyaluronic acid *in vitro* will be tested by the mucin clot method.

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Calcification XIX. Calcification of Transplanted Rachitic Bone.*† (23416)

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The purpose of this investigation was to study calcification of rachitic bone slices that were transplanted subcutaneously to rats with the thought that transplantation would be a useful approach to the study of the calcifying mechanism as was the case with *in vitro* calcification(1-10). By standardizing both the rachitic sections and the host animal, a transplantation technic would permit the study of calcification after experimental modification of either the rachitic section or the composition of body fluid in the host animal.

Experimental. Method of preparation of animals for the transplants. Each animal was

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anesthetized with ether and placed on its back. The skin covering the abdomen was cleansed with 70% alcohol and a 12 to 15 mm incision was made along the medial ventral line about 20 mm anterior to the genital organ. All instruments and sutures were sterilized prior to use and sterile precautions were adhered to in the handling of the transplants. After the incision was made, the transplant was placed into a subcutaneous pocket by lifting the skin and pushing the transplant with a blunt end forceps laterally from the line of incision to a distance of about 20 mm. The incision was closed with 3 or 4 stitches. Each animal was housed in a separate cage for the duration of the experiment. Antibiotics were not used and in only one instance was infection encountered.

Two groups of experiments were carried out. An inbred strain of Wistar rats was used.

Group one consisted of transplanting rachitic tibial slices into rats that were 6 to 8 weeks of age. Half of the rats were rachitic and half were normal. The rachitogenic diet was composed of 75% degerminated corn meal, 19% wheat gluten, 2% Brewer's yeast, 3% calcium carbonate and 1% sodium chloride. The normal diet was that of Bills *et al.* (11).

The tibial slices used for transplantation and for *in vitro* calcification were obtained from rats that had been maintained on the aforementioned rachitogenic diet for 3 to 4 weeks after weaning and were prepared, under sterile precautions, as previously reported (12). Three, frontal plane and longitudinally sliced sections were obtained from each tibia. One section was used to test for rickets using the silver line test as described in the next paragraph. The second section was transplanted to a rat that had been fed the rachitogenic diet for 3 weeks after weaning and the third section was transplanted to a rat that had been fed the normal diet for 3 to 4 weeks after weaning. Separate transplants were made to at least 5 individual animals on each of the diets.

The following procedure was used to test for rickets. A section was placed in a concavity of a glass spot plate, covered with 3% silver nitrate solution and exposed to light from a 100 watt tungsten filament lamp for 5 minutes. The distance from the section to the lamp was about 8 inches. After exposure, the section was examined with a Leitz wide field stereobinocular microscope at a magnification of 32 X. If healing was observed, as evidenced by a black silver stain in the rachitic metaphysis, the sections from that animal were discarded.

A similar procedure (line test) was used to determine degree of calcification of each of the experimental sections in this study. The evaluation and the procedural details have been described by Sobel *et al.*(13,14).

The slices from the other tibia were subjected to *in vitro* calcification for 18 hours as described previously(9,14). The *in vitro* calcifying solution was prepared by adding to a one liter volumetric flask, 100 ml of a stock basal salt solution‡ and 50 ml of a NaH_2PO_4 - Na_2HPO_4 solution that had a phosphorus concentration of 1 mg/ml. Five hundred ml of glass distilled water was added and the pH lowered to about 6.3 by aerating with 100% CO_2. To this solution were added either 2 ml or 5 ml of specially prepared calcium chloride solution that had a calcium concentration of 20 mg/ml. Thus, the calcifying media had Ca x P products of 20 and 50, *i.e.*, 4 mg% Ca x 5 mg% P, or 10 mg% Ca x 5 mg% P. Distilled water was added to the liter mark. The pH of the final *in vitro* calcifying solution was adjusted to 7.3 by aerating with 100% N_2.

In addition to the *in vitro* calcification in purely inorganic media, *in vitro* calcification of tibial slices was performed in sera obtained from rachitic and from normal rats. Blood from partially anesthetized animals was obtained from the severed jugular vein and collected in test tubes that contained 3 ml of sterile mineral oil. The tubes were centrifuged and the sera aspirated, pooled and thoroughly mixed. The pooled sera, maintained under oil at all times had a pH of 7.32. The calcium content was determined by the method of Sobel and Sobel(15) and the phos-

‡ The stock basal salt solution contained 0.7 M NaCl, 0.5 M KCl and 0.22 M $NaHCO_3$/l. It was Berkefeld filtered and stored in a pyrex bottle.

phorus by the Fiske and Subbarow method (16). The pooled sera from the rachitic rats and the pooled sera from the normal rats were aseptically transferred in four 10 ml portions for each of the sera to 12.5 ml capacity plastic capped vials. Mineral oil was added to fill each vial and the cap was kept in place by the use of rubber bands stretched tightly over the cap and under the bottom of the vial. After incubation at 37°C for 18 hours, the cap was removed and the mineral oil aspirated off. The tibial slice was washed with distilled water and subjected to the silver line test.

In one of the experiments, pooled rachitic serum was divided into 2 portions. One portion, unmodified, had a Ca x P product of 15.9. The other portion was modified by increasing the phosphorus content from 1.5 mg% to 5.0 mg% by the addition of inorganic phosphate. Thus, the Ca x P product of this serum was increased from 15.9 to 53.0. A rachitic slice was suspended in each of 2 vials containing 10 ml of the unmodified serum and in like manner, a rachitic slice was suspended in each of 2 vials containing 10 ml of the phosphorus enriched serum. A layer of mineral oil was placed over the serum and each vial was capped and incubated as mentioned above. The degree of *in vitro* calcification (as the average of 2 slices for each serum) is shown in Table II.

In the second group of experiments, 20 slices of rachitic tibiae were divided into 2 portions. One portion, consisting of 10 slices placed separately into 10 sterile test tubes of 15 ml capacity and stoppered with sterile rubber stoppers was shaken mechanically for one hour in 150 meq/l $CaCl_2$ at 24-26°C. The calcium chloride solution was Berkefeld filtered just prior to use. After shaking, the slices were washed with freshly distilled water to remove the excess of calcium chloride. The second portion was not treated with calcium chloride. Both portions were transferred to sterile vials and stored for 2 weeks at −22°C ± 2° in a deep freeze chest. After the 2 week period of storage, one calcium treated and one untreated slice was implanted under the skin of each animal laterally and on opposite sides of the medial ventral line incision. The animals were 7 to 8 weeks of age and had been maintained on the normal diet. After 1, 2 or 3 weeks following the transplantations, the animals were sacrificed by chloroform asphyxiation. The transplants were removed and examined for degree of calcification in the rachitic metaphysis. The *in vitro* calcification of similarly prepared deep freeze stored tibial slices was carried out as described previously(17). The *in vitro* calcifying solution had a Ca x P product of 50 (10 mg% Ca x 5 mg% P).

Results. There was no evidence of calcification of the bone transplants that were placed into the rats on the rachitogenic diet. In contrast, the transplants in the rats on the normal diet showed a progressive increase in calcification from week to week and a simultaneous decrease in the width of the uncalcified epiphyseal cartilage. After one week the calcification in the provisional zone of the hypertrophic cartilage was 1.3(+++). After 2 weeks the calcification was 2.5(++++) and after 3 weeks 3.5(++++). All of the transplants in the animals on the normal diet were encapsulated with fibrous tissue. Removal of the capsule revealed an extremely hard transplant which could not be readily cut with a scalpel. This was especially true for the transplants that were placed into the animals for 2 and 3 weeks. In all instances the metaphyseal osteoid was well calcified too. On several occasions, ridges similar in hardness to compact bone developed along the edges of the slices. This material produced a dense, black silver stain when immersed in silver nitrate solution and exposed to light. The transplants excised from the rats on the rachitogenic diet were less encapsulated and removal of the capsule revealed a soft transplant which could be easily cut with a scalpel.

Here we have an example of one way in which transplantation can be used in calcification studies. The difference in calcification of the same type of rachitic bone is due to the differences in the body fluid brought about by different diets. In these studies it was possible to establish that the predominant factor that altered calcification was the Ca x P product of the serum, 16.7 for the rachitic animals and 89.8 for the normal animals (Table I).

TABLE I. Calcification of Preosseous Cartilage of Rachitic Tibial Slices Transplanted to Rats on Rachitogenic and Normal Diets.

Diet	Blood serum Ca, mg %	Blood serum P, mg %	Ca × P product	Degree of calcification after 1 wk	2 wk	3 wk
				Mean		
Rachitogenic	9.8	1.7	16.7	0 (0)	0 (0)	0 (0)
Normal	10.2	8.8	89.8	1.3(+++)*	2.5(++++)*	3.5(++++)*

* Calcification was observed in the metaphyseal osteoid and was most extensive after 3 wk.

Table II summarizes the *in vitro* calcification results. When the serum of rachitic animals was reinforced by added phosphate, thus raising the Ca x P product, calcification *in vitro* occurred just as it did in the serum of normal animals. With unmodified rachitic serum *in vitro* calcification did not occur. This is parallel to the findings of *in vitro* calcification in inorganic media where calcification took place at the higher product.

TABLE II. Calcification of Preosseous Cartilage of Rachitic Tibial Slices *In Vitro* for 18 Hours in Inorganic Medium and in Serum.

Ca × P product	Ca, mg %	P, mg %	Degree of calcification
	Mean		
Inorganic medium			
20	4.0	5.0	0 (0)
50	10.0	5.0	1.5(++++)
Rachitic rat serum			
15.9	10.6	1.5	0 (0)
53.0	10.6	5.0*	1.1 (+++)

* The phosphorus in this serum was raised from 1.5 mg % to 5.0 mg % by the addition of inorganic phosphate.

In this manner, factors other than the serum Ca x P product favoring or retarding calcification may be detected in the future. For example, animals under the influence of drugs, hormones or hereditary differences may contain these factors.

In the second group of experiments in which deep freeze stored bone slices, with and without prior treatment with calcium chloride were placed into animals on a normal diet, the treated slices showed a more rapid rate of calcification in the rachitic metaphysis. The results are shown in Table III. The treated transplants excised after one week in the animals showed 1.4(++++) calcification in the epiphyseal cartilage while the untreated transplants showed only a trace. After 2 weeks, the calcium treated transplants were not only more densely calcified than the untreated transplants but marked calcification was observed in the metaphyseal osteoid in addition to that in the epiphyseal cartilage. After 3 weeks, the untreated transplants still showed a wider uncalcified epiphyseal cartilage as compared to the calcium treated transplants. The latter showed a 3. (++++) calcification in the epiphyseal cartilage and extensive calcification in the metaphyseal osteoid while the untreated transplants showed a 1.5(++++) calcification in the epiphyseal cartilage and only traces in the metaphyseal osteoid.

Since the animals in this experiment were fed the same normal diet and since each animal received one calcium treated and one untreated transplant the difference in degree of calcification of the transplants may be as-

TABLE III. Calcification of Preosseous Cartilage of Transplanted Rachitic Tibial Slices Treated and Untreated with Calcium Chloride Prior to Deep Freeze Storage at −22°C ± 2° for 2 Weeks.

Treatment of slices prior to deep freeze storage	Degree of calcification after: 1 wk	2 wk	3 wk
	Mean		
None	.7 (+)	.9 (+++)	1.5 (++++)
Shaken 1 hr at 24-26°C in 150 meq/l of $CaCl_2$	1.4 (++++)	2.3 (++++)*	3.0 (++++)*

* Extensive calcification was observed in the metaphyseal osteoid.

TABLE IV. *In Vitro* Calcification of Preosseous Cartilage of Rachitic Tibial Slices Treated and Untreated with Calcium Chloride Prior to Deep Freeze Storage for 2 Weeks.

Treatment of slices prior to deep freeze storage	Degree of calcification after 18 hr (Ca × P = 50)
	Mean
None	0 (0)
Shaken 1 hr in 150 meq/l $CaCl_2$	1.5 (++++)
Fresh, untreated and not deep frozen	2.0 (++++)
Fresh, unfrozen, shaken 1 hr in 150 meq/l $CaCl_2$	2.5 (++++)

cribed to the differences in the bone rather than the body fluid. The *in vitro* calcification studies in pure inorganic solution showed that the deep freeze (not calcium treated) stored slices would not calcify at a Ca x P product of 50 in solutions containing 5 mg% P either as inorganic or organic phosphorus while the calcium treated slices calcified at this product (Table IV). It is apparent from these findings that the calcifying mechanism is inactivated in the untreated deep freeze stored bones and this inactivation can be prevented or reversed with Ca ions(17). The transplants showed a partial restoration of the calcifying mechanism in the deep freeze stored bone slices which may indicate that possibly the body fluids contain some ingredient which, if present in the *in vitro* fluid, might have been manifested in calcifiability.

These studies with deep freeze stored bones exemplify the influence on the calcifying mechanism of bone treated in various ways and which may be tested under conditions that exist in a standard body fluid, such as that of a normal animal. This method is not limited only to rachitic bone taken out and treated thereafter but could conceivably use excised rachitic bone produced in different ways such as in Sr rickets(5), Be rickets(4) or under the influence of hormones, such as parathormone or rickets produced in animals by thyroid-parathyroidectomy(18). Testing such bones only by *in vitro* calcification may miss significant information that may be discovered by transplantation.

The usefulness of transplantation is indicated in some of our other studies with demineralized bone, with and without chondroitin sulfate treatment, and treated in various ways(19) which showed that calcification will occur where *in vitro* calcification does not appear to occur (to be published elsewhere). Another study showed that ATP plus Ca^{++} treated bones calcified *in vitro* at lower products(20,21) and mineralized when transplanted to a rachitic animal in which the animal's own bones would not mineralize.

Summary. Calcification of the rachitic metaphysis, with typical silver line test, was obtained in tibial slices that were transplanted to rats on a normal diet. Similar transplants to rats on a rachitogenic diet did not calcify. This difference was probably due to the higher Ca x P product in the body fluids of animals on a normal diet as compared to the product of animals on a rachitogenic diet. *In vitro* calcification was obtained in the sera of normal animals, in the sera of rachitic animals to which phosphate was added to raise the Ca x P product but not in the unmodified sera of rachitic animals. *In vitro* calcification was obtained in inorganic solutions with a Ca x P product of 50 but not with a Ca x P product of 20. Transplanted deep freeze stored bone slices treated with calcium chloride prior to deep freeze calcified more intensively than deep freeze stored bones not treated with calcium, as measured by the silver line test. Calcification *in vitro* studies indicated reversible inactivation of calcifiability of stored frozen bone which could be prevented or restored by calcium chloride treatment. While differences existed, calcification of the deep freeze stored bone indicated that there was a partial restoration of the calcifying mechanism or that some unknown ingredient exists in the body fluids which, if present, could have elicited restoration of the calcifying mechanism. Transplantation of rachitic bone, as proposed, may be an additional method for studying the differences of the actual body fluids on calcification of a given rachitic bone, or after treatment of such bone the changes in calcifiability with an actual standard body fluid such as that of an animal on a normal diet.

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Antihemophilic Factor (AHF): Plasma Levels After Administration of AHF Preparations to Hemophilic Dogs.* (23417)

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Intravenous administration of plasma fractions rich in antihemophilic factor (AHF) has been shown to be followed by a rapid rise in the plasma level of AHF. In normal and hemophilic dogs, half of the plasma AHF was lost in 2-6 hours(1,2). A similar rise and fall in AHF was observed in human hemophiliacs following injections of large volumes of plasma(2) or bovine AHF fractions(3). Recently it was shown that high plasma AHF levels occurred following intravenous injection of a human plasma fraction(4). Similar quantitative studies on other routes of administration of AHF are not available, although many years ago it was observed(5,6) that the clotting time in hemophilia was shortened after intramuscular injections of a plasma globulin substance. Other workers (7,8) indicated subsequently that intramuscular injections were both ineffective and dangerous. Data presented in this paper demonstrate the relative effectiveness of various routes of administration of a plasma AHF fraction to hemophilic dogs. After each injection, plasma tides of AHF were determined. A preliminary report of some of these data was given earlier(9).

Materials and methods. Lyophilized bovine AHF was prepared by a modification of Bidwell's procedure(10). On the basis of protein and AHF determinations the prepara-

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