L. STUDIES UPON CALCIFICATION *IN VITRO.*

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The object of these experiments was to determine the conditions under which calcification occurs *in vitro*, and also what factors interfere with calcification. If it can be shown that the position and the character of the calcification, in other words the histological picture, are exactly the same as those occurring *in vivo* then it would seem justifiable to draw certain deductions regarding *in vivo* calcification from *in vitro* experiments. *Per contra*, if certain factors prevent calcification *in vitro*, and if it can be shown that these same factors are operative in diseases such as rickets, in which there is a failure of calcification, then it would appear justifiable to explain the failure of calcification by the influence of these factors.

The basis for these experiments was the observation of Shipley [1924] that, when the cartilage and bone of a rachitic rat are placed in the serum of rachitic rats and incubated for a number of hours, no calcification occurs. On the other hand, when a piece of cartilage and bone from the same animal is placed in the serum of normal rats and incubated for the same length of time, calcification, similar to that taking place in an animal undergoing cure, can be demonstrated. It is obvious that this method is applicable to the study of various aspects of calcification and for this it has been employed.

In previous studies of rickets from this department it has been shown that, in uncomplicated rickets, there is regularly a low concentration of inorganic phosphorus in the serum, 1·5 to 3·5 mg. per 100 cc., as opposed to the normal of 5 to 6 mg. Studies in conjunction with the School of Hygiene have demonstrated that rats can be rendered rachitic by feeding them a diet very low in calcium and relatively high in phosphorus or high in calcium and low in phosphorus (McCollum's 3143 diet). The low phosphorus diet is the most satisfactory one to use, for it produces a very severe rickets with great regularity. The low phosphorus in the diet is reflected in the low inorganic phosphorus of the serum and, instead of the normal 8 mg. of inorganic phosphorus in the serum, the rachitic rat has usually 2·5 to 3 mg. per 100 cc. It has further

been shown that, when as the result of some therapeutic procedure the inorganic phosphorus concentration of the serum rises, evidences of reparative processes appear in the bones. The position and the character of these reparative processes are perfectly definite and regular.

The information thus available determined the conditions under which we carried out the experiments especially as regards the concentration of the substances in the solutions employed. Certain precautions must be rigidly observed. The rats must be completely rachitic, *i.e.* there must be no trace whatever of calcification in the epiphyseal cartilages. Before utilising the bones of an animal we have stained a slice of bone and cartilage in order to determine freedom from calcification. If any calcification was present the animal was rejected. The animals must eat well up to the time they are killed. It has been shown that, if rachitic animals are starved for a period, the inorganic phosphorus of the serum rises and calcification of the cartilage begins. In some of the experiments of this series it has seemed that, if animals were deprived of food even for a few hours, the cartilage calcified more readily than does that of an animal which has been well fed. The animals must be killed without anaesthesia, by a blow on the head. The slices of tissue must not be too thin for reasons that will appear later. Aseptic precautions must be observed throughout the experiment.

Our procedure has been as follows. After the animal was killed, the skin was removed from a leg and with a sterile, small knife the muscles were removed and the knee joint disarticulated. The upper end of the tibia was isolated and slices about 0·5 to 0·75 mm. thick containing the centre of ossification, the epiphyseal cartilage, the metaphysis and a little of the shaft were made. One slice was examined for the presence of calcification. If satisfactory, the other slices from the same bone were immersed in solutions in 25 cc. Erlenmeyer flasks. The solutions had been previously prepared and warmed to 37°. After adding the tissue the flasks were immediately placed in an incubator and maintained at 37°. The solutions were made up in a number of different ways but all of them contained about 0·03 % sodium bicarbonate, $N/10$ NaCl and 0·0016 N $MgSO_4$. The calcium was varied from 0·0025 to 0·005 N (5·0 to 10·0 mg. per 100 cc.) and the inorganic phosphorus from 0·0015 to 0·009 N (1·5 to 9·0 mg. per 100 cc.). To each litre of solution 20 cc. of a 0·03 % sol. of phenolsulphonephthalein were added as an indicator. Certain solutions contained egg-albumin and some a colloid, gum arabic. The calcium salt in most of the solutions was the chloride, but the lactate, acetate and citrate were also employed.

All the components save the calcium were added and the solution was charged with carbon dioxide. The calcium salt was then added and water to the desired volume. The solution was filtered through a Berkefeld candle and the p_H adjusted by equilibration with CO_2 to reactions which were approximately physiological. The solutions were warmed, poured into 25 cc. sterile Erlenmeyer flasks and incubated for a few hours before use. No anti-

septics were used except when it was desired to study the effect of these upon calcification. The specimens were examined, usually at the end of 8 hours, and at intervals thereafter, sometimes as late as 2 weeks. At this time the p_H of the solution was read by comparison with phosphate standards and the temperature was determined. The tissue was removed, washed with distilled water, immersed in a 1 % solution of silver nitrate and examined under a binocular microscope with direct illumination.

When aqueous solutions were used we have sometimes set a time limit of 9 hours for the first examination. If at the end of this time calcification had not occurred we have considered the experiment negative. But this is purely arbitrary. In experiments with a normal calcium concentration (10 mg. per 100 cc.) and with a low concentration of phosphorus, calcification will occasionally occur at the end of 20 to 48 hours but with no regularity and the calcification is slight. Calcification regularly occurs in 9 hours when the concentrations of calcium and of inorganic phosphorus are 10 and 4 mg. respectively per 100 cc. This is shown in Table I.

Table I. *Calcification in aqueous solutions of inorganic salts.*

Ca mg. per 100 cc.	P mg. per 100 cc.	Precipitate	Calcification	p_H	Temp. ° C.	Time hours
10	1·0	0	0	7·42	35	9
10	2·0	0	0	7·35	35	9
10	3·0	0	0	7·45	35	9
10	4·0	0	+ + +	7·42	35	9

When the concentration of phosphorus is 3·5 mg. it requires 16 to 24 hours for a similar degree of calcification to take place. It is interesting to note that the result is the same whether the calcium is 10 mg. and the phosphorus 3·5 or the calcium 5 and the phosphorus 7 (Table II). The same process occurs with more concentrated solutions and very rapidly. We have obtained it with 9 mg. of phosphorus.

Calcification occurs best when no precipitation whatever takes place in the flask, for the obvious reason that precipitation lowers the concentration of calcium and phosphorus. Experiments are best made, therefore, at a p_H of 7·1 to 7·3 but the calcification will occur through even a wider range of reaction (Table II).

Table II. *Effect of reaction on calcification.*

Ca mg. per 100 cc.	P mg. per 100 cc.	Precipitate	Calcification	p_H	Time hours
10·0	3·5	0	+ + + +	7·05	24
10·0	3·5	0	+ + + +	7·10	24
5·0	7·0	0	+ + + +	7·00	24
5·0	7·0	0	+ + + +	7·10	24
10·0	3·5	0	+ + +	7·48	24
10·0	3·5	0	+ + +	7·50	24

In the above-mentioned experiments calcium chloride was used. We have made similar observations using the lactate, acetate and citrate in the same concentration of calcium (10 mg.) and with a concentration of inorganic phosphorus of 6 mg. per 100 cc. With the acetate and lactate, calcification was as prompt and thorough in 9 hours as with the chloride. With the citrate there was none even at the end of 48 hours.

The process of calcification is completely inhibited by certain substances. Previous soaking in chloroform, ether, 95 % alcohol, potassium cyanide (10 %), formalin (10 %), or toluene, or previously boiling the piece of tissue prevents calcification. A few drops of toluene added to the fluid prevented calcification in one experiment. The process does not occur in the cold (ice box).

Having established the fact that calcification regularly occurs in 24 hours with a phosphorus concentration of 3·5 mg. and in 9 hours with a concentration of 4·0 mg., we studied the effect of the addition of protein (1 and 2 %) to the solution. The protein employed was egg-albumin (Merck's soluble) which contained neither calcium nor inorganic phosphorus in sufficient amount to influence the results. The experiments are recorded in Tables III and IV.

Table III. *Effect of protein* (1 %) *on calcification.*

Ca, 10 mg. per 100 cc.; P, 3·5 mg. per 100 cc.

Precipitate	Calcification	p_H	Temp. ° C.	Time hours	Albumin %
0	+ + +	7·40	36	16	0
0	+ + + +	7·40	37	24	0
0	+ + + +	7·36	36	48	0
0	0	7·40	37	16	1
0	0	7·38	37	24	1
0	0	7·30	36	48	1

Table IV. *Effect of protein* (1 *and* 2 %) *on calcification.*

Ca mg. per 100 cc.	P mg. per 100 cc.	Precipitate	Calcification	p_H	Temp. ° C.	Time hours	Albumin %
10	4·0	0	+ + +	7·30	36·5	19	0
10	4·0	0	+ + +	7·35	36·5	19	1
10	4·0	0	0	7·25	37·0	26	2
10	4·0	0	0	7·25	37·0	47	2
8	5·0	0	+ + + +	7·45	36·5	7	0
8	5·0	0	0	7·20	37·0	42	2

The albumin clearly exerted a retarding influence, which was more marked in the higher concentration. With a 2 % albumin concentration no calcification occurred in 47 hours when the calcium was 10·0 and the phosphorus 4·0 mg. per 100 cc. A 4 % solution of gum arabic also had a striking inhibitory effect. That this failure of calcification was not due to injury of the tissue was demonstrated by the fact that the tissue at the end of 24 hours could be removed, washed and placed in an aqueous solution of the same inorganic concentration, when calcification promptly occurred.

We have repeated Shipley's original observation but have used human serum from normal and rachitic children instead of rat's serum. Calcification occurred in 19 to 48 hours in normal or relatively normal serum whereas in the rachitic serum it did not occur in the same interval of time.

Table V. *Calcification* in vitro *with human serum.*

Ca mg. per 100 cc.	P mg. per 100 cc.	Calcification	Time hours
	Non-rachitic serum.		
10·4	5·2	+	19
10·0	4·3	+	19
9·8	4·4	+	19
7·8	6·5	+	48
10·0	5·0	+ + +	20
	Rachitic serum.		
10·6	3·2	0	48
10·0	2·5	0	48
10·5	3·7	0	19

We have had an opportunity to make observations with human cartilage from two cases, one in a child who died suddenly and unexpectedly while convalescent from scurvy, and the other in an infant with severe rachitic changes in the thorax. In each instance the rickets was unusually severe. Pieces of costochondral junctions were placed in a solution with a concentration of calcium and of inorganic phosphorus of 10·0 and 6·0 mg. per 100 cc. respectively. Calcification occurred in less than 18 hours and was extensive in all the eight specimens examined.

When healing occurs in a bone affected by rickets calcification may come about in either of two ways. If recalcification goes on in response to a powerful stimulus, lime salts are laid down in the cartilage and a new zone of provisional calcification is formed before there is histological evidence of new calcification in the rachitic metaphysis. On the other hand, under certain conditions provisional calcification of the cartilage may be retarded and calcium phosphate may be deposited in the cartilage and the metaphysis simultaneously. In some cases the metaphysis begins to calcify before calcification of the cartilage is complete and the former process may be well under way before the latter is begun. The above is true for the healing of rickets in man or in the rat.

Calcification *in vitro* proceeds in the first mentioned manner and the first event is the formation of a line of calcification in the proliferative cartilage. As is the case when rickets heals in man, the calcification does not begin uniformly throughout the provisional zone but in small areas separated from each other by uncalcified cartilage, which, as the process proceeds, becomes calcified in such a manner that the interrupted line of calcification becomes a continuous one (Plate I, fig. 1).

The calcium phosphate is deposited in the intercellular matrix and never in the cytoplasm of the cells themselves, just as is the case when healing

occurs *in vivo* in the rat or in the body of the child. As a result of the freedom of the cells from calcification the calcified matrix has the appearance of a honey-comb (Plate I, figs. 2 and 3). Calcification *in vivo* or *in vitro* does not extend through the matrix of the entire zone of proliferative cartilage but is limited to the shaftward portion (Plate I, figs. 2 and 3). It is sharply delineated on the epiphyseal side and terminates in a straight or gently curved line (Plate I, figs. 2 and 5). The irregular tongues of cartilage which dip down into the metaphysis also become calcified.

Calcification does not begin on the surface of the slice of tissue as one might expect *a priori*, but the first deposits of lime salts are in the depths of the cartilage and the process gradually extends to the surface. It is for this reason that it is unwise to have the slices of tissue too thin, for, if they are, the calcification may be irregular or nearly wanting. It appears as if the trauma of cutting interfered somewhat with the integrity of the cartilage.

Celloidin sections from pieces of cartilage and bone calcified *in vitro* have the identical appearance of sections of cartilage and bone calcified *in vivo*. The calcified matrix has a granular appearance but there are no deposits in or on the cells themselves, when the sections are properly made and stained (Plate I, figs. 4 and 5).

Numerous attempts have been made to produce calcification in pieces of dead cartilage. Among them may be mentioned those of Pfaundler [1904], Wells [1911], Freudenburg [1922], etc. These experiments were made with the object of proving by chemical analysis the impregnation of the tissues with lime salts and have, therefore, no relation to our own work. More recently Robison and his co-workers [Robison, 1923; Robison and Soames, 1924; Kay and Robison, 1924] state that they have produced calcification of cartilage and bone *in vitro* by the action of an esterase upon solutions of calcium and phosphorus in the form of calcium hexosemonophosphate and calcium glycerophosphate. The concentrations of calcium and phosphorus were much higher than those found in normal serum and the reaction far more alkaline than is believed to be possible in the human body (p_H 8·4 to 9·4). Robison obtained uniformly negative results with concentrations of calcium and phosphorus corresponding to those found in normal serum and also negative results with higher concentrations when no esterase was added.

The calcification that was present in our experiments with inorganic salts as well as those with the calcium salts of lactic and acetic acids did not resemble the calcification pictured in the article by Robison and Soames. In our experiments the deposition of calcium was sharply limited to the intercellular matrix between the cells of the provisional zone (Plate I, figs. 3 and 5). In many instances the intercellular matrix of this whole zone was completely calcified. There was no granular deposit in the cells or in the resting cartilage. A granular deposit was found on the surface of the slices a few times when the solution was strongly alkaline and a precipitate had formed in the solution, but when a precipitate had formed calcification was usually incomplete

or absent. In all the experiments that we have recorded in the beginning of this paper calcification was unaccompanied by precipitation.

Robison has suggested an ingenious theory to explain calcification. Briefly this theory is as follows. Calcium and phosphorus exist in the blood and presumably in the tissue fluids in part as calcium hexosemonophosphate, in part as tertiary calcium phosphate. By means of this distribution they are enabled to remain in solution in serum at their high, normal concentration. Normal as well as rachitic cartilage and bone and also other tissues contain an esterase which liberates inorganic phosphorus from its organic hexose combination. Tertiary calcium phosphate is formed in excess of its solubility and therefore precipitates. Before accepting this theory there are certain aspects of the whole problem that should be considered. Do the calcium and phosphorus of the serum exist to a significant amount as calcium hexosemonophosphate? Robison himself has been unable to answer this question in the affirmative. He has, however, demonstrated the presence in red blood cells of an organic phosphorus compound which liberates inorganic phosphorus upon the addition of esterase[1]. We observed several years ago that when the serum of normal or rachitic children or animals is allowed to stand at 37° in contact with the clot the inorganic phosphorus of the serum rapidly rises. The extent of this increase is shown in Table VI.

Table VI. *Effect on inorganic phosphorus of serum of standing in contact with clot.*

(Temperature 37°.)

	Mg. per 100 cc.				Mg. per 100 cc.		
	Before	After incubation	Time hours		Before	After incubation	Time hours
Rat	3·5	6·2	24	Infant	6·6	20·0	24
„	2·9	10·3	24	„	3·3	16·0	24
„	2·9	6·2	24	„	3·4	16·0	24
„	7·0	24·0	24	„	3·4	12·0	24
„	2·8	11·2	24	„	3·5	15·0	72
„	6·0	10·4	24	„	2·2	13·2	24
„	7·0	13·0	24	„	6·0	18·0	24

For this reason we have insisted upon the necessity of prompt separation of the serum. Obviously, therefore, the conversion of organically bound phosphorus and the diffusion of the latter from the cells into the plasma can occur *in vitro* even in the absence of esterase. We have shown that, if inorganic phosphorus and calcium are present in such concentrations as in normal serum, calcification promptly occurs without the addition of esterase other than that which might be present in the slice of tissue undergoing calcification. If esterase plays a determining rôle in calcification why is it present in large amounts in rachitic as well as in normal cartilage and in tissues that are the seat of calcification only under exceptional circumstances? We have found

[1] However, it is unlikely that the organic compound of the red blood corpuscles is a hexosemonophosphate, for the ratio of hexose to phosphorus in the barium salt that he isolated is not that which obtains in barium hexosemonophosphate.

that the immersion of slices of tissue in antiseptics or the use of these in the solutions definitely inhibits or entirely prevents calcification. Robison's experiments have been done with antiseptics such as chloroform. For this reason it does not seem remarkable to us that the calcification which Robison obtained should have been inconstant and incomplete.

The reaction (p_H) at which Robison's experiments were carried out (p_H 8·4–9·4) is one which can hardly be considered to obtain in the body and in our own experiments has been found to interfere with, rather than to facilitate, calcification.

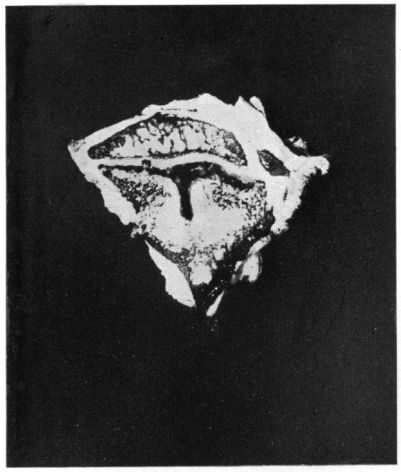
The illustrations in the article by Robison and Soames resemble the diffuse deposition of calcium phosphate from very alkaline solutions rather than the calcification which takes place *in vivo* in the process of healing. Granular deposits over the cells themselves may be formed during the process of staining sections if this technique is improperly carried out.

The fact that *in vitro* calcification occurs in simple solutions of inorganic salts including calcium and phosphate, with or without protein, at a temperature and reaction and in concentrations of calcium and phosphorus which may be supposed to exist in the animal body, and that this calcification is similar in all respects to the calcification which occurs *in vivo*, makes it unnecessary to assume the local action of an esterase upon a substrate of calcium hexosemonophosphate. In fact it is unlikely that such an action can play a rôle in calcification since the calcium and phosphate which reach the cartilage cells already exist in sufficient concentration for calcification in inorganic form. This is true in the serum and presumably the tissue fluids of normal children. In rachitic children the concentrations are insufficient.

It is not to be denied that such a process as Robison describes might explain the liberation of inorganic phosphorus from the red blood cells but there seems to be no special necessity for assuming that this takes place almost entirely in the bones and cartilage, or to postulate the necessity of an enzyme to bring this about since it can be shown that under conditions identical with those described by Robison liberation of inorganic phosphorus from the corpuscles occurs, without the addition of enzyme.

Lehnerdt [1910] in his excellent article upon the failure of calcification in rachitic bones came to the conclusion that this failure was due, not to a primary deficiency of calcium in the diet or to an abnormal metabolism producing an increased excretion of calcium but to an inability of the newly formed bone to incorporate into itself the circulating calcium. It is obvious that Lehnerdt, and many others, regarded the primary disturbance as one chiefly associated with calcium. The fact that the calcium concentration of the serum in uncomplicated rickets is often quite normal or reduced only to a slight extent, whereas the inorganic phosphorus concentration is regularly much lower than normal, indicates that the primary disturbance concerns the phosphorus.

It has been shown by the experiments recorded above that the bones and



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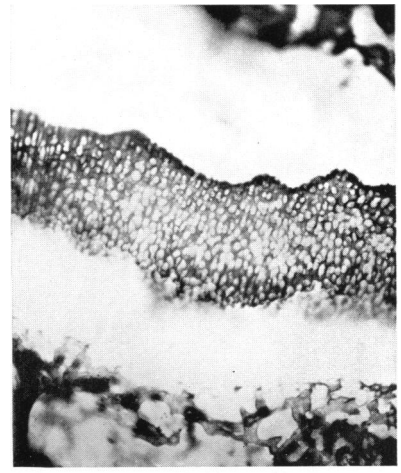

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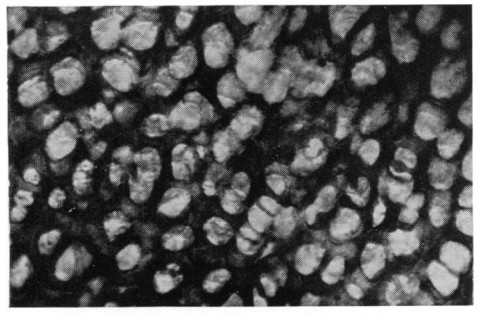

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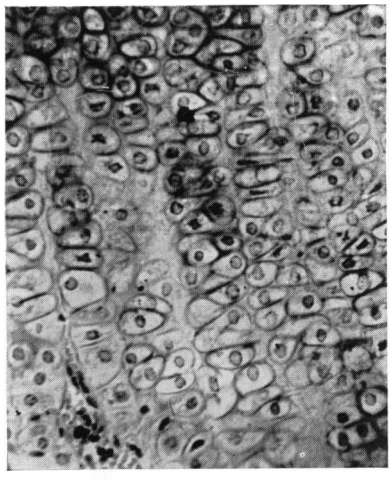

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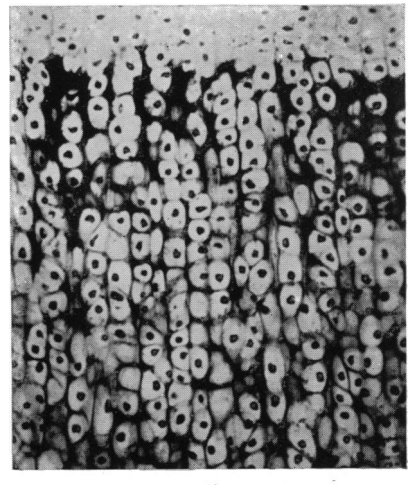

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cartilage are perfectly capable of undergoing calcification provided the concentrations of phosphorus and calcium are adequate, whether in simple inorganic solutions, in solutions with albumin, or in relatively normal serum. They will not undergo calcification if the concentration of inorganic elements is insufficient. The conclusion is inevitable that the disturbance is not primarily in the bones. They do not calcify because the substances necessary for this do not reach them in sufficient concentration.

SUMMARY.

Calcification occurs *in vitro* apparently exactly as it does *in vivo*. In aqueous solutions it occurs rather rapidly only when the concentration of the bone-forming constituents and the reaction are nearly those of normal serum. Calcification is delayed by the addition of egg-albumin and completely inhibited by heat, by cold and by protoplasmic poisons.

It is impossible to advance any theory to explain the selective deposition of the calcium salts. The process is clearly not one of simple precipitation. It depends upon the activity of living tissue. It cannot occur unless the concentration of calcium and phosphorus in the serum and presumably in the fluid bathing the cells exceeds a certain minimal value.

The failure of calcification in rickets does not depend upon any primary inability of rachitic bone or cartilage to undergo calcification. The failure depends upon an insufficient concentration of the necessary elements in the fluids bathing these tissues.

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DESCRIPTION OF PLATE I.

Fig. 1. Proximal end of the tibia removed from a rachitic rat and kept for 19 hours in a solution of salts containing 10 mg. of calcium and 4 mg. of inorganic phosphorus per 100 cc. The newly deposited lime salts were blackened with silver nitrate. × 8.

Fig. 2. Photomicrograph of a part of the newly formed provisional zone of calcification. The specimen from which this was taken had been dehydrated and cleared in oil of wintergreen after treatment with nitrate of silver. × 85.

Fig. 3. This and the above figure show the honeycomb-like appearance of the provisional zone of calcification. This figure, taken from a slice of bone prepared as described above, shows that the calcification is confined to the intercellular matrix and does not involve the cell bodies. × 400.

Figs. 4 and 5. These photographs were made from celloidin sections (stained H. and E. and silver nitrate) of slices of bone taken from a costochondral junction of a rachitic child who had died 18 hours before *post mortem* examination. The tissue photographed for Fig. 5 had been kept 24 hours in a solution of salts containing calcium 10 mg. and inorganic phosphorus 4 mg. per 100 cc. The blackened deposits of lime salts are in the cartilage matrix between the cells. Fig. 4 was taken from a specimen fixed at autopsy and shows the cartilage without a trace of calcification. × 350.