milliequivalents per liter or less) were noted in 3 cases, but no significant change could be detected in hematocrit, carbon dioxide content, chloride, sodium, protein or urea nitrogen concentration of the serum. There was no major alteration in the excretion of sodium, potassium or chloride in the urine. Determinations of serum volume with the blue dye T. 1824 were carried out in 2 subjects before and at the end of the period of extract injection with no significant change. Ballistocardiographic tracings were unaltered in one patient who exhibited a drop in blood pressure. Toward the close of the extract period there was no leucocytosis, eosinophilia or rise in erythrocyte sedimentation rate; no effect on the electrocardiogram or teleroentgenogram; and the patients complained of no symptoms during the treatment period other than the headache previously mentioned.

Discussion. In the dosage employed, the continued administration of adrenal cortical extract was associated with a decrease in "resting" blood pressure in 3 of 4 hypertensive patients. The changes in arterial tension could not be ascribed to alterations in serum volume, fluid or electrolytes, or apparent change in cardiac action.

The results suggest the possibilities that the fall in blood pressure may reflect suppression of adrenal cortical function or represent counteraction of a desoxycorticosterone-like pressor hormone. The adrenal cortex may possess a homeostatic group of substances concerned with blood pressure regulation.[3] However, one cannot exclude the possibility that the decrease in tension observed in this study may have been the result of some non-specific component contained in the extract. Further search for depressor materials is indicated among purified products of the adrenal cortex.

Summary. The continued administration of an adrenal cortical extract for one month, to four subjects with uncomplicated hypertensive vascular disease on a constant regimen, was associated with a decrease in "resting" blood pressure in 3 instances, a small rise in arterial tension in one.

[3] Soffer, L. J., *Diseases of the Adrenals*, Lea & Febiger, Philadelphia, 1946.

16459

Influence of pH on Growth of Bone in Tissue Culture.*

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Although a great amount of work has been done on the general subject of bone growth, the exact actions of the factors involved are poorly understood. The problem of bone growth and pH is a case in point. With little or no experimental evidence the idea is widespread that the calcification which accompanies bone growth occurs because of a local change to a more alkaline pH. To ascertain whether or not this speculation was correct we undertook to grow bones in tissue culture at two levels of pH, namely 7.0-7.3 and 7.8-8.0.

The pertinent literature on the problem traces largely from the work of Robison and his co-workers. Following Robison's discovery[1] of phosphatase in bone the theory was advanced that this enzyme played an important role in ossification. Further work revealed that in the test tube phosphatase was most active between pH 8.4 and 9.4 when glycerophosphate was employed as the substrate.[2] Robison then postulated that osteo-

* Supported by a grant from the United States Public Health Service. The author is also indebted to Miss Marie T. Coppola for her technical assistance.

[1] Robison, R., *Biochem. J.*, 1923, **17**, 286.

[2] Robison, R., and Soames, K. M., *Biochem. J.*, 1924, **18**, 740.

blasts and hypertrophic cartilage cells could raise the pH of tissue fluid around them. The effect of this on a saturated solution of $Ca_3(PO_4)_2$ and $CaCO_3$ would be to produce precipitation of the salts. However it was not considered essential that the pH be raised as high as 8.4; a small change would cause the desired effect.[3]

The above findings and opinions were used by Fell[4] to explain, in part, the slowness of ossification in tissue culture as compared with the greater speed of the process *in vivo* since the average pH of the medium in which tissue is growing is more acidic than is the pH of normal blood plasma.

Others have subscribed to the theory[5,6] and even extend it to explain calcification in degenerating cartilage. As chondrocytes degenerate, metabolic activity drops, fewer metabolites are produced, the pH rises and calcification is thereby favored.[6]

Howland[7] was more specific. He visualized the calcification process involved in bone growth as depending on CO_2 tension. The presence of a relatively high CO_2 level due for example to cellular activity is unfavorable to deposition of bone salts but in an inactive tissue such as bone and cartilage "one would expect the CO_2 tension to be low and it is in them that precipitation takes place."

Direct experimental observations do not support the idea that a local increase in pH favors bone formation.[8] The early work of Rous[9] with indicators injected into the living animal revealed pH's in bone of 6.5 to 7.2. Pierce[10] made direct observations on the pH of epiphyseal cartilage in tibias of rachitic and normal rats. The bones were split and laid open like a book. Using a special quinhydrone electrode mounted on a micromanipulator, he obtained no detectable pH differences between resting and proliferating cartilage. Studies[11] on the ion products of calcium phosphates in normal serum with changes in pH led to the conclusion that any serum which does not cause calcification (presumably of cartilage slices) at the pH of the blood will not do so when the pH is raised to 8.0.

As regards the cultivation of bone in tissue culture it seems almost superfluous to mention the excellent and widely quoted works of Fell.[4,12,13]

Method. In growing bones in tissue culture a technic modified after that of Fell[14] was used. Vials that held 32 cc were used instead of Petri dishes and watch glasses. Each vial received 15 drops of Tyrode solution containing phenol red, 15 drops of embryonic chick extract and 15 drops of blood plasma. After the media were thoroughly mixed the vials were laid horizontally and left unmolested while the clot formed. The vials were taped to a U-shaped piece of metal rod to prevent rolling.

Femurs were dissected from 7 day chick embryos and those from one animal placed in marked areas of separate vials (Fig. 1). Routinely 4 femurs were put in each container. In each experiment a minimum of 16 femurs were used.

The pH of the media was controlled by the continuous flow of gases through the vials. A two-holed paraffined cork supporting an inlet and an outlet tube was employed instead of the usual blind cork stopper (Fig. 1). Each tube had a cotton filter. A continuous stream of the desired gas passed into the vial through one tube and out of the other. Two vials received air with no CO_2 at the rate of about one liter of gas for each vial per day. The pH of these vials as read colorimetrically against standard tubes was 7.8-8.0. Another two

[3] Robison, R., *Biochem. J.*, 1926, **20**, 388.

[4] Fell, H. B., *Arch. f. Exp. Zellforsch.*, 1929, **7**, 390.

[5] Howard, J. E., Conference on Bone and Wound Healing, 1st Meeting, p. 26, N.Y., 1942 (Josiah Macy, Jr., Found.).

[6] Weinman, J. P., and Sicher, H., *Bone and Bones*, pp. 40-43, C. V. Mosby Co., St. Louis, 1947.

[7] Howland, J., *Medicine*, 1923, **2**, 349.

[8] McLean, F. C., *Ann. Rev. Physiol.*, 1943, **5**, 79.

[9] Rous, P., *J. Exp. Med.*, 1925, **41**, 739.

[10] Pierce, J. A., *J. Biol. Chem.*, 1938, **124**, 115.

[11] McLean, F. C., Conference on Bone and Wound Healing, 14th Meeting, pp. 9-20, New York, N.Y., 1946 (Josiah Macy, Jr., Found.).

[12] Fell, H. B., *Arch. f. Exp. Zellforsch.*, 1931, **11**, 245.

[13] Fell, H. B., *J. Morph. and Physiol.*, 1925, **40**, 417.

[14] Fell, H. B., *J. Roy. Mic. Soc.*, 1940, **60**, 95.

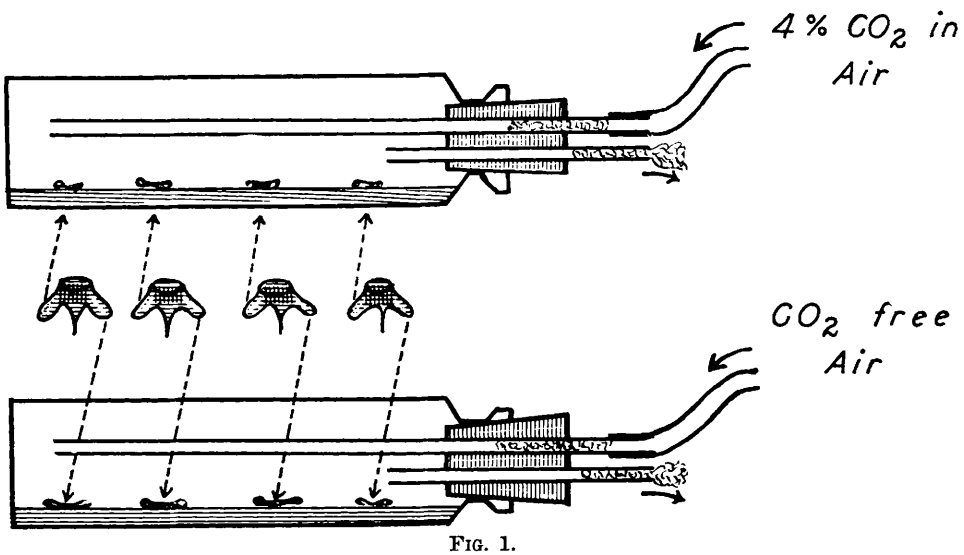

FIG. 1.

vials containing mates to the femurs in the first were flushed continuously with air containing 4% of CO_2, one liter per vial each day. The pH of these was 7.0-7.3 as read colorimetrically.

The femurs were transplanted every second or third day as deemed necessary. Cultivation in every case was arbitrarily terminated after 14 days. At this time 8 pairs of femurs were fixed in formaldehyde, embedded in paraffin, sectioned serially and stained with hematoxylin and eosin. Projection drawings were made of the areas in which bone had formed. Each bone was projected at the same magnification, and the relative amount of bone grown in each femur was determined by the paper weight method. In a second group of femurs the calcium was determined quantitatively. The femurs were wet ashed with sulfuric acid and 30% H_2O_2, neutralized, made up to a volume of 2 cc, and the calcium determined on aliquots by the method of Sobel and Sobel.[15]

Observations. Embryonic femurs growing in tissue culture look like femurs. Growth is organized. When planted at the 7th day of embryonic development the femurs consist entirely of cartilage and are quite small. At the end of 14 days of cultivation in the vials there has been a marked increase in size. In terms of normal development, however, the amount of growth occurring in 14 days of cultivation is equivalent to a scant 3 days of normal growth in the embryo.

As seen in section the cultivated femurs consist principally of cartilage with a well-formed zone of bone around the shaft. In hematoxylin-eosin preparations the bone appears as a distinct red band.

In Table I the relative amounts of bone

TABLE I.

The Relative Amounts of Bone Grown in 11 Pairs of Femurs. One of each pair was grown at pH 7.8-8.0; the other at pH 7.0-7.3. The amount of bone is expressed in terms of per cent. Bone growth at pH 7.8-8.0 was 100% in every test.

Tubes	Pair of femurs	Bone grown at pH 7.8-8.0	Bone grown at pH 7.0-7.3
1 and 2	1	100	111
	2	100	128
	3	100	90
	4	—	—
3 and 4	5	100	209
	6	100	134
	7	100	92
	8	100	197
5 and 6	9	100	171
	10	100	96
	11	100	113
	12	100	183
Avg		100	139

[15] Sobel, A. E., and Sobel, B. A., *J. Biol. Chem.*, 1939, **129**, 721.

TABLE II.
The Calcium Content of 8 Pairs of Femurs. One of each pair was grown at pH 7.8-8.0; the other at pH 7.0-7.3.

Tubes	Pair of femurs	Calcium in femurs grown at pH 7.8-8.0	Calcium in femurs grown at pH 7.0-7.3
A and B	1	43 γ	170 γ
	2	67 γ	54 γ
	3	63 γ	233 γ
	4	18 γ	116 γ
C and D	5	74 γ	166 γ
	6	46 γ	61 γ
	7	61 γ	98 γ
	8	39 γ	79 γ
Avg		51 γ	122 γ

expressed as per cent are shown for each member of 11 pairs of 7 day femurs after 14 days of growth in tissue culture. No data are presented for the fourth pair because one of them was accidentally fractured in transplanting to fresh media. Of the remaining 11 pairs of femurs it will be seen that in 8 of them the ones grown at pH 7.0-7.3 had distinctly more bone than their mates grown at the more alkaline pH of 7.8-8.0. Only 3 of the 11 pairs of femurs grown in the more alkaline medium contained more bone than did those grown at pH 7.0-7.3. The averages reveal that at a pH of 7.0-7.3 the amount of bone was approximately 40% more than at the more alkaline pH of 7.8-8.0.

In Table II the total amount of calcium present in each member of 8 pairs of femurs is listed. In 7 of them the bones growing at pH 7.0-7.3 contained distinctly more calcium than did their respective mates growing at the more alkaline pH of 7.8-8.0. In only one pair, the second, was there more calcium deposited at pH 7.8-8.0 than at 7.0-7.3. The average values indicate a marked increase in calcium in those femurs cultivated at pH 7.0-7.3.

Discussion. Before discussing the above observations let us recall what is meant by "the second calcifying mechanism" or "the local factor." According to the phosphatase theory of calcification the enzyme acts on organic phosphates to liberate phosphate ions which react in turn with calcium ions to produce a form of calcium phosphate. The calcium phosphate is deposited in areas where ossification is occurring. But why does not calcification occur in all places where phosphatase, organic phosphates and calcium ions are found, as *e.g.*, in the wall of the intestine? It is thought that a second mechanism, some local factor, must be present before calcification will occur. Such a factor is not present in the intestinal wall but is found in areas where bone formation is progressing. There are various theories as to just what the local factor is. As indicated above many people regard it as being a local change toward a more alkaline pH.

Our results, however, are not in agreement with the concept that bone formation in a given area is favored by a local change in pH toward the alkaline. The pH of chicken blood is about 7.6 yet when the femurs are grown at a pH of 7.0-7.3 about 40% more bone is found on the average than when the femurs are held at a pH of 7.8-8.0. In addition the calcium determinations reveal that the average calcium content of femurs grown at pH 7.0-7.3 was 122γ but only 51γ in femurs grown at pH 7.8-8.0. For 2 weeks the femurs lay on the surface of a clot and were exposed directly to the atmosphere which controlled the pH. Considering that the bones were isolated it seems reasonable to assume that as regards pH the local factor was well controlled. Bearing these facts in mind the statement is entirely justified that an increase in alkalinity from 7.0-7.3 to 7.8-8.0 favors neither bone formation nor calcium deposition in bones growing in tissue culture.

Objections may arise on the basis that the paper weight method of determining the amount of bone is crude. The method is admittedly not very accurate in that we are dealing with small amounts of bone and the subjective element is a formidable one. The method is however a practical one and was used to distinguish the amount of bone formation and calcification. The bone is distinguishable in the stained preparations as a red zone bearing the same positional relationship to the underlying cartilage as a napkin ring to the napkin. While the 39%

average difference may not be accurate, a difference in the amount of bone existed and a smaller amount was found in the more alkaline medium.

The values for calcium need qualification. It is not implied that they represent only the calcium deposited in the bone. Instead the entire amount of calcium deposited throughout the life of the femurs, *i.e.*, 7 days *in ovo* and the additional 14 days of cultivation in tissue culture, was measured. Moreover, the calcified area is not restricted to the zone of ossification, but involves portions of the cartilage as well. Yet, based on the assumption that one femur contains the same amount of calcium as its mate when first planted in tissue culture, the statement is entirely reasonable that the difference in the calcium values for each member of the 8 pairs listed in Table II is due to variations in pH over the 2 weeks of cultivation *in vitro*.

The quantity of calcium and phosphate available in the media upon which the bones grew was not determined. It was presumed to be adequate since the media consisted of adult chicken blood plasma, embryo extract made from 10 to 11 day chick embryos and Tyrode solution. The precaution was taken to use the same stock media for both the low and high pH cultures.

The most intriguing part of the problem concerns the enzyme phosphatase. From the work of Fell and Robison[16] it is known that not only is it synthesized in the bones grown in tissue culture but it is present in amounts larger per unit weight than can be found in bones *in vivo*. Phosphatase is the key component of the phosphatase theory of bone formation. In the hands of the chemist[17] the activity of the enzyme "rises rapidly from pH 7.0 to pH 9.0: *e.g.* at pH 7.5 the activity is double that at pH 7.0 and at pH 8.0 more than fourfold the value at pH 7.0." If it is assumed that the amount of the bone formed and the amount of calcium deposited is an indication of phosphatase activity then why is less bone formed at a high pH than at a low one? At a pH of 7.8-8.0 the enzyme should be from 2 to 4 times as active as at a pH of 7.0-7.3.

While we do not agree with the implied doubt of Shipley, Kramer and Howland[18] and later McLean[11] that phosphatase plays a key role in the calcifying mechanism, we did not expect the results which we obtained. The pH 7.8-8.0 is one which will support general growth of embryonic chick tissue very well (Paff).[19] From the day by day appearance of the bones growing in culture one could not observe any gross difference between femurs grown at a low and at a high pH, yet they did differ. Perhaps the activity pattern of phosphatase in the living tissue differs from the pattern followed by the enzyme in the simpler systems not involving live tissue.

The observations are conclusively in favor of discarding the idea that the local factor in bone growth is a change in pH toward a more alkaline reaction. In bone growing in tissue culture it can be said that if a change in pH is desirable then it is in the direction of less, not more, alkalinity. By decreasing the pH perhaps more nonionized calcium is ionized making it more readily available for reaction with phosphate. Since PO_4 ions are more numerous at the site of phosphatase activity, *i.e.*, in areas where osteoblasts are found, it should be in this region that more bone would be produced.

Our results are the exact opposite of those which would be predicted on the basis of Howland's CO_2 theory.[8]

Summary and Conclusions. Seven day chick embryo femurs were grown in tissue culture for 2 weeks at 2 pH levels, *i.e.*, pH 7.0-7.3 and 7.8-8.0. The pH's were maintained by a continuous flow of gas, 4% CO_2 in air for the low level and air minus CO_2 for the high. Our results show that more bone is formed and more calcium is deposited at the low than at the high pH. If a local change

[16] Fell, H. B., and Robison, R., *Biochem. J.*, 1929, **23**, 767.

[17] Borne, G., *Cytology and Cell Physiology*, p. 219, Oxford Univ. Press, 1941.

[18] Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, 1926, **26**, 379.

[19] Paff, G. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 184.

in pH is desirable in bone growing in tissue culture then it is in the direction of less rather than more alkalinity. These data are strong evidence against the widespread belief that the local factor in bone growth is a change in pH toward the alkaline.

16460

Response of the Syrian Hamster to the Virus of Newcastle Disease.

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The adaptation of the California strain of Newcastle disease virus No. 11,914 to the Syrian hamster through 12 intracerebral passages has been reported.[1] This Newcastle virus has been passed through 22 successive series of 10- to 12-day embryonated chicken eggs prior to hamster injection. Intracerebral culture in hamsters has been continued through 200 uninterrupted passages.

Infected hamsters used for passage were sacrificed while moribund. Suspensions were prepared by grinding the infected brain tissue in a mortar with alundum and diluting with physiological saline. The inoculum for the first 50 successive passages was approximately 0.08 cc of a 10% (10^{-1}) suspension; for the 51st to the 120th passage 0.03 cc of suspension of the same concentration was used; and after the 150th passage each hamster was inoculated with 0.03 cc of a 1% (10^{-2}) suspension.

The incubation period in the first 12 passages extended from 2 to 6 days. In these early passages symptoms of irritability followed by paralysis developed slowly over a period of approximately 2 days, and these hamsters remained alive for at least one day after becoming paralyzed. Approximately 50% of the inoculated hamsters of the first 8 passages showed irritability and increased sensitivity, followed by involuntary motor reactions and paralysis, before becoming moribund. After the eighth passage all inoculated hamsters developed the above-mentioned typical signs of central nervous system involvement.

From the 70th through the 120th passages, signs of central nervous system involvement were observed approximately 36 hours following inoculation, and most of the hamsters became moribund in 48 hours. From the 160th passage through the 190th passage the hamsters showed signs of central nervous system involvement in 24 hours and died within 36 hours of the inoculation. After the 190th passage infected hamsters showed signs of central nervous system involvement in 12 hours and 75% died within 24 hours.

Hamster brain suspensions from the 49th and 72nd passages titrated 10^{-3} by intracerebral inoculation in 4-week-old hamsters. The infected brain suspensions of the 200th passage titrated $10^{-4.25}$ in 4-week-old hamsters and $10^{-3.5}$ in adult hamsters, as is shown in the table.

Neither the egg-adapted nor the hamster-adapted Newcastle disease virus produced symptoms of disease in hamsters when injected subcutaneously, intramuscularly, intradermally, or intraperitoneally.[2] The hamster-adapted Newcastle disease virus after the 92nd passage was not pathogenic for chickens between 3 and 6 weeks of age when injected intramuscularly or subcutaneously.

[1] Reagan, Reginald L., Lillie, Mary G., Poelma, Leo J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 136.

[2] Reagan, Reginald L., Lillie, Mary G., Poelma, Leo J., and Brueckner, A. L., *Am. J. Vet. Res.*, 1947, **8**, 136.

[3] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.