dermis-subcutaneous tissue interface has to be considered. Decreased circulation does not seem likely, however, as the vessels are capillary in type, vertically oriented, and relatively profuse. No change in color or evidence of necrosis was observed following surgical manipulation. The possibility that repeated manipulation also might be interfering with the vertically oriented (dermal-subdermal) interface led to modification of the secondary wounding procedure in Group III rats. We reasoned that, by creating a single large secondary wound on either side of an advancing primary wound margin, the horizontally oriented cell mass would be disrupted more effectively and with less vertical damage than Group II wounds. More significant reduction in the rate of movement of such isolated segments strongly suggests that horizontal cell mass integrity has a measurable influence on the rate of movement of the wound edge.

The rate of secondary wound contraction (Group IV) was measured to document and confirm the impressions of Grillo that secondary manipulation of primary contracting wounds does not cause an acceleration of the contracting process similar to the accelerated rate of gain in tensile strength seen in incised and sutured secondary wounds(2,3).

Summary and conclusions. Repeated radial incisions which interrupt the horizontal integrity of the "picture frame" area of specialized cells in a full-thickness wound significantly decrease the rate of contraction of the wound edges. The rate of movement of a segment of advancing wound edge is retarded significantly by partially isolating such segments from the horizontally oriented picture frame cell mass. Rate of secondary wound contraction is not significantly different from rate of primary wound contraction. These data suggest that physical integrity of a horizontally oriented cell mass in the "picture frame" area of a contracting full-thickness skin wound exerts a measurable influence on rate of the contracting process.

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Culture Medium for Suitable Growth and Development of Embryonic Chick Femora *in vitro*.*† (29631)

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This laboratory has previously used a natural medium for cultivation of embryonic chick femora which consisted of rooster serum and embryo extract in a 3:1 ratio(1). It is clear that any attempt to define the hormonal and nutritional factors regulating growth in this organ culture has to begin with a definition of the medium. Biggers and his colleagues have taken a synthetic approach to the establishment of such a medium, adding compounds in a stepwise fashion (2,3,4). We have taken as our goal the identification of necessary growth factors in the natural medium.

Eagle and Peiz(5) have shown that in the presence of an adequate level of known water-soluble nutrients (minerals, amino acids, vitamins) only very small amounts of the protein synthesized by HeLa cell cultures were derived from protein in the medium. This suggested that we could replace the serum in the femur culture medium with one

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of the defined media. We chose as our serum substitute Waymouth's MB 752/1, because at the time these studies began, it was the simplest completely defined medium which would support mammalian cell growth(6). We very early found that MB 752/1 would not support growth at a rate comparable to the natural medium unless embryo extract was added. However, MB 752/1 with embryo extract appears adequate without the addition of serum.

Materials and methods. The explant. Femora were carefully dissected from 7-day White Leghorn chick embryos and gently rolled on filter paper moistened with a rinsing solution to remove any adhering soft tissue. Only those femora pairs were used for culture whose lengths fell within the range of 2.56 mm-2.88 mm using an ocular micrometer.

Culture media. Waymouth's MB 752/1(6) was modified slightly and used as the basic synthetic medium for all of the control cultures. MB 752/1 is prepared from 3 stock solutions, S_1, S_2, and S_3. S_1 contains salts, glucose, ascorbic acid, cysteine, glutathione, choline, hypoxanthine and glutamine. S_2 contains water soluble vitamins and S_3 contains amino acids. For better pH control we used a CO_2 incubator. The original bicarbonate concentration of the medium was too high to give a pH of 7.4 upon equilibration with the 5% CO_2-95% air mixture. Therefore the sodium bicarbonate in S_1 was decreased so that the final medium contained 123.5 mg per 100 ml. We omitted the $MgCl_2$ from S_1 and adjusted the $MgSO_4$ concentration to give an equivalent total magnesium ion concentration. As described later, the magnesium and calcium levels were changed in the course of the work. The composition of the modified S_1 is given in Table I. MB 752/1a is the modified medium with the additional alteration in magnesium and calcium ion levels. The 3 stock solutions are sterilized by passage through Selas No. 03 filters. Solutions S_1 and S_2 can be stored indefinitely in a deep freeze while S_3 keeps indefinitely at +4°C.

MB 752/1 is then composed of 50 ml of S_1, 2.5 ml of S_2, 10 ml of S_3 and 37.5 ml deionized water which makes a total volume of 100 ml. It was convenient to add extra materials to MB 752/1 by merely substituting them for the water without changing the concentration of any of the other components. Various levels of embryo extract and rooster serum were added to MB 752/1 in this manner. The culture medium varied with each experimental culture as shown in Table II. With careful technique it was possible to maintain sterile cultures without use of antibiotics in the medium.

TABLE I. Composition of the S_1 Component of MB 752/1, Modified MB 752/1 and MB 752/1a. The concentrations given are those in the complete medium.

	MB 752/1	Modified MB 752/1	MB 752/1a
m group	mg/100 ml		
NaCl	600.0	600.0	600.0
KCl	15.0	15.0	15.0
$CaCl_2$	9.1	9.1	22.2
$MgCl_2 \cdot 6H_2O$	24.0		
$MgSO_4 \cdot 7H_2O$	20.0	49.0	35.8
Na_2HPO_4	30.0	30.0	30.0
KH_2PO_4	8.0	8.0	8.0
$NaHCO_3$	224.0	123.5	123.5
Glucose	500.0	500.0	500.0
n group			
Ascorbic acid	1.75	1.75	1.75
Cysteine · HCl	9.0	9.0	9.0
Glutathione	1.5	1.5	1.5
Choline · HCl	25.0	25.0	25.0
Hypoxanthine	2.5	2.5	2.5
Glutamine	35.0	35.0	35.0
Phenol red	2.0	2.0	2.0

Embryo extract (EE) was prepared from 10-day chick embryos as a 50% solution. The embryos were forced through the barrel of a sterile 50 ml syringe into a large graduated centrifuge bottle. An equal volume of sterile 0.9% NaCl was added and the mixture allowed to stand overnight at +4°C. After centrifugation at 2000 rpm for 30 minutes, the EE was decanted into sterile bottles and stored up to 6 weeks in a deep freeze.

Rooster serum was prepared from whole blood obtained by sterile heart puncture. Serum from several roosters was pooled and stored in a deep freeze.

Culture technique. The roller-tube method of culture was used throughout these studies (7,8). Small rectangles of washed lens paper were placed near the bottoms of the tubes

and one femur placed on the lens paper in each tube. The lens paper washing procedure was carried out in a paper chromatography jar. A book of lens paper with the metal staple removed was mounted in the jar so that the washing solution dripped off the paper. The washing solution was 1% tetrasodium ethylenediaminetetraacetate. A liter of this solution was followed by distilled water until the washings were neutral. The paper was then washed with ethanol, which facilitated drying.

The femora were kept in pairs as they were dissected from the embryos and were placed in the culture tubes so that one femur of the pair was incubated in one milliliter of the control medium while the other was incubated in one milliliter of the experimental medium. The tubes were capped with Morton closures(9), and placed in a roller drum (12 rph) slanted at a 5° angle in a 38°C incubator containing a 5% carbon dioxide-95% air mixture which was water saturated. In every case the culture period was 8 days, with the medium being changed every other day.

Analytical methods. Dry weight. The femora were removed from culture, quickly rinsed in deionized water, dried for 3 hours in a 100°C oven and then weighed directly on a Sartorius micro balance.

Calcium. After obtaining the dry weight of each femur, it was placed in a small ground glass homogenizer (10 mm × 75 mm) and homogenized with 200 μl of 5% trichloracetic acid. The pestle was rinsed with 100 μl of 5% TCA and removed, after which the homogenizer tube was centrifuged. The supernatant containing extracted calcium was removed and the precipitate extracted once more with 200 μl of 5% TCA. The pooled supernatants were evaporated to dryness overnight in a 165°C oven, in order to concentrate the calcium within the range of the method. The dried supernatant was dissolved in 50 μl of 5% TCA, and the calcium content quantitatively determined by microtitration using a calcein indicator. The method was essentially that supplied with the Beckman/Spinco 153 Microtitrator(10), but with slight changes as described by Wetherell(11) and Mori(12).

Statistical treatment. The data obtained from cultures 1-8 (Table II) were statistically analyzed using the Student's T-test, comparing the means obtained at each level with the mean obtained from the culture grown with 25% EE (No. 6). The data obtained from cultures 10-18 (paired cultures) were analyzed by the paired variate method using the Student's T-test(13).

Results. The results are shown in Table II. Cultures No. 1 through 8 are unpaired, and show the growth achieved with varying amounts of EE added. The average weight of a 7-day femur is about 100 μg. The 36% increase in MB 752/1 alone was not considered satisfactory, since in serum:EE (3:1) we have obtained dry weights of about 800 μg(1). There was a 400% increase in dry weight with 15, 20 and 25% EE, with no significant difference between the means for these levels ($P>0.1$). The dry weight with 30% EE, however, was significantly lower ($P<0.01$). From these results it was decided to use MB 752/1 + 25% EE as the control medium for the remainder of this study.

Beginning with culture No. 8, it was decided to determine the calcium content of each femur, as an index of differentiation. The average calcium content of 7-day femora was found to be 0.987 μg. After 8 days in culture this had only increased to 1.808 μg. For reasons which will be explained later, it was decided to increase Ca^{++} concentrations and lower Mg^{++} concentration of MB 752/1. Ca^{++} was increased from 3.2 mg% to 8.0 mg% while Mg^{++} was decreased from 4.8 mg% to 3.0 mg% (Table I). Normal chicken serum contains about 11.2 mg% Ca^{++} and 2.7 mg% Mg^{++}(14). As can be seen from the calcium content of the controls in the subsequent cultures, these alterations more than doubled the amount of calcium deposited.

Cultures No. 9-18 are paired cultures, where the control femora were grown in MB 752/1a + 25% EE. These experiments were performed in order to determine, first, if all

TABLE II. Dry Weight and Calcium Content of 7-Day Embryonic Chick Femora Cultivated for 8 Days in Various Media.

Culture No.	Description of culture	Dry wt (μg)			Calcium (μg)			Ca^{++} (μg/mg dry wt)		
		Control	Exp	P	Control	Exp	P	Control	Exp	P
1	MB 752/1 + 0% EE		136.6	.01						
2	*Idem* + 5% EE		246.3	.01						
3	" + 10% EE		240.5	.01						
4	" + 15% EE		427.4	.05						
5	" + 20% EE		444.1	.05						
6	" + 25% EE		468.8							
7	" + 30% EE		391.9	.01						
8	" + 25% EE		616.3			1.08			1.82	
9	C *vs* S_{1m} + 25% EE	652.0	220.5	.01						
10	C *vs* S_1 + 25% EE	567.5	292.4	.01						
11	C *vs* S_1 + S_2 + 25% EE	499.9	295.8	.01	2.73	2.57	.1	5.67	8.62	.05
12	C *vs* S_1 + S_3 + 25% EE	461.8	841.5	.01	2.24	2.77	.1	4.76	3.41	.1
13	C *vs* S_1 + S_3 + 25% EE	908.6	876.5	.1	10.55	9.78	.1	11.66	11.27	.1
14	C *vs* S_{1m} + S_3 + 25% EE	810.0	405.4	.01	9.98	1.12	.01	12.40	2.87	.01
15	C *vs* S_{1m} + S_2 + S_3 + 25% EE	876.7	397.3	.01	2.42	1.85	.1	2.70	4.63	.01
16	C *vs* C + 10% serum	857.4	781.2	.05	2.69	2.78	.1	3.26	3.61	.1
17	C *vs* C + 1% "	821.5	913.4	.1	3.07	2.12	.1	3.74	2.34	.01
18	C *vs* C + 5% "	812.8	834.7	.1	2.76	2.77	.1	3.36	3.32	.1

C is control medium containing MB 752/1 + 25% EE. Beginning with Culture No. 9, the $CaCl_2$ content of MB 752/1 was increased to 22.2 mg/100 ml and $MgSO_4 \cdot 7H_2O$ lowered to 35.8 mg/100 ml. Beginning with Culture No. 13, a freshly prepared stock solution of S_2 was used in preparing the culture media. All values are the avg of 10 to 12 bones. EE added at levels indicated was a 50% solution.

of the components of MB 752/1a were necessary in the presence of EE, and secondly if serum benefited the cultures. S_{1m} consists of the salts in S_1 plus glucose (Table I). As shown by the results in Table II, there is a significant difference in dry weight between controls and experimentals in Cultures No. 9, 10 and 11. This indicates that neither S_{1m}, total S_1 nor S_1 plus S_2 can substitute for the complete MB 752/1a. This shows a requirement for S_3, which contains the amino acids.

When S_2 was omitted in Culture No. 12, a significant increase in dry weight was obtained. S_2 is the vitamin mixture, which had been stored at 4°C for a little over a year. Evidently deterioration had occurred with the production of toxic products. When the experiment was repeated with fresh S_2 (Culture No. 13) no difference was observed with the omission of the vitamins. Apparently the EE contains all of these vitamins.

Cultures No. 14 and 15 demonstrate a significant decrease in dry weight when the more labile compounds in S_1 are omitted (S_{1n}, Table I). Except for culture No. 14, the calcium content of the femora in this group did not show any significant change over the controls.

The results from cultures No. 16, 17 and 18 show no significant difference in dry weight or in calcium content with the addition of 1, 5 or 10% serum to MB 752/1a + 25% EE.

Discussion. As pointed out by Neuman and Tytell(15), although embryo extract and serum have been eliminated from the growth requirements for mammalian cells, this has not been done for avian tissues. It is not surprising therefore that specific requirements for avian organ cultures are still not completely known. MB 752/1 supports about a 36% increase in dry weight of 7-day chick embryo femora after 8 days in culture. However, supplementation of MB 752/1 with embryo extract achieves a 600 to 800% increase in dry weight.

The studies of Fell and her colleagues have shown that cultured avian embryonic bones undergo a normal differentiation in addition to an increase in mass(16). Osteoblasts differentiate from the perichondrium and elaborate a calcifiable matrix (osteoid) which mineralizes. We decided to use the appearance of this mineral as an index of differentiation and the total calcium content of the femora was used as a quantitative measure of mineralization. We found that 7-day femora had an average calcium content of 0.987 μg per bone.

After 8 days culture in MB 752/1 + 25% EE the bones contained 1.080 μg. Clearly mineralization was not increasing. This could be due to an inadequate differentiation of osteoblasts with a consequent lack of mineralizable matrix, or the matrix might be present but the medium was inadequate to support mineralization. MB 752/1 contains a comparatively high level of Mg^{++}, which served to promote cell adhesion in the mammalian cell cultures for which it was designed(6). High levels of Mg^{++} however, increase the apparent solubility of the bone mineral(17). Changing the concentrations of Ca^{++} and Mg^{++} in the medium to those present in chicken serum did produce an appreciable increase in the calcium content of the femora.

Our results from the calcium analyses were not consistent when expressed either in content per bone or per mg dry weight. We suspected that the uncontrolled factor(s) stem from changes in the embryo extract during storage. Miszurski made some observations on cultures of 7-day chick embryo tibiae which suggests that embryo extract contains 2 factors, one of which stimulated growth while the other stimulated differentiation (18). It is altogether possible that these factors are inactivated at deep freeze temperatures, at different rates. This might also explain the great variation in dry weight of the controls.

We were particularly impressed by the lack of significant effect of serum upon either weight or calcium content. Endo(8) and Ito *et al*(19), have studied the growth and differentiation of 9-day chick embryo femora in a medium composed of embryo extract, horse serum and Gey's saline. They found the optimal ratio of these components was 1:5:4. From their histological studies they concluded that EE was necessary for osteoblast maintenance, while serum favored the proliferation and hypertrophy of cartilage cells. From our quantitative determinations, we do not find that serum improves growth or differentiation in the presence of the nutrients in MB 752/1a.

Although there appears to be no requirement for the water-soluble vitamins, in the presence of embryo extract, we have chosen to retain them in the medium. This was done in order to facilitate the discovery of new growth factors in the embryo extract.

Summary. We have found that Waymouth's medium MB 752/1, when modified (MB 752/1a has a higher level of calcium and a lower level of magnesium), plus embryo extract will support the growth and differentiation of cultured 7-day embryonic chick femora. The optimum level of embryo extract was 15 to 25% of the medium, and 25% was routinely used. In this medium the femora attain an 8-fold increase in dry weight and a 3-fold increase in calcium content during an 8-day culture period. Addition of chicken serum at 1, 5 or 10% of the medium did not improve these results.

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Alkaline Phosphatase and Osteogenesis *in vitro.**† (29632)

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The discovery by Robison(1) of a "monophosphoric esterase" in ossifying cartilage of young rats and rabbits led the way to further studies of this enzyme and its role in osteogenesis. This "bone phosphatase" has been found to belong to a group of phosphatases having activity at a pH optimum around 10.0 and is referred to as alkaline phosphatase. The present investigation is concerned with the alkaline phosphatase of bone and its participation in ossification and also in calcification of cartilage and bone.

The original phosphatase theory of calcification as proposed by Robison(1) has been seriously questioned, and several other roles have been assigned to this enzyme. Various studies have shown alkaline phosphatase to be involved: (a) in differentiation of both osteoblasts and chondroblasts from their mesenchymal origin(3), (b) in formation of fibrous protein and the pre-osseous matrix of bone(4), (c) in formation of mucopolysaccharides of the ground substance(5), (d) in transphosphorylation of some phosphate acceptor in the matrix(6), and (e) in removal of organic phosphates that may poison bone crystal growth(7).

Many investigators have used either normal embryonic and post fetal bone or rachitic bone slices for the study of alkaline phosphatase in osteogenesis. However, since the classical experiments of Fell and Robison (2) in 1929, impetus was given to the use of tissue culture as a tool for demonstrating and study osteogenesis of embryonic bone tissue *in vitro.*

Material and methods: Tissue culture technique. Seven-day embryonic chick femora (White Leghorn), were grown in culture on plasma clots using the roller-tube technique. The culture medium contained 3 parts of chicken serum and one part of 50% embryo extract. Each day the embryo extract was prepared fresh according to the procedure described by White(8) and using Tyrode's solution(9) as the diluent. The chicken serum was prepared from whole blood obtained by sterile heart puncture using 2- to 3-month-old White Leghorn roosters. It was stored at 4°C for use within 2 to 3 weeks. Chicken plasma was obtained in the same manner and prepared using heparin as the anticoagulant. Each pair of femora was placed in a roller-tube (16 mm × 150 mm), end to end and lengthwise to the tube. One drop of plasma was allowed to run down over the pair of femora; in this manner a very thin plasma clot was formed, due to the small amount of embryo extract still on the femora. After approximately 10 minutes, 0.80 ml of culture medium was added to each roller-tube. The tubes were stoppered with white rubber stoppers and placed in a roller drum, set at 5° angle in a 38°C incubator, and revolving at 12 rph. The culture medium was changed every two days. Sterility was maintained without the use of antibiotics.

Lyophilization and dry weight measurement. At the end of the culture period for each series of cultures, the paired femora were removed from their respective roller-

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