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Endogenous Inhibitor of Bone Cell Proliferation (41032)

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Abstract. A substance recovered from the medium of embryonic chick bones growing in organ culture inhibits the proliferation of chick calvarial bone cells *in vitro*. This inhibition of proliferation occurs within 3 hr after exposure of the cells to the inhibitory substance (IS) in a manner which does not appear to affect the protein synthesis or the gross morphology of the cells. The medium from which IS is obtained can be diluted 500-fold and still retain significant inhibitory activity. Cells in culture spontaneously escape the inhibition of a single dose of IS within 24 hr of exposure, however, upon further addition of IS to these cells inhibition of proliferation is again observed. Cells derived from chick bone as well as rat and human bone are more sensitive to the inhibition than are chick dermal fibroblasts and chick liver and muscle cells. IS is not destroyed after treatment of the conditioned medium with trypsin, phospholipase A_2, RNase, neuraminidase, or heat. IS is also not a commonly produced prostaglandin. Estimates of the molecular weight of IS range from 6000 to 14,000 daltons. While these features do not disclose the exact nature of this substance, they are consistent with this substance being a small polypeptide without the exposed amino acids, lysine or arginine. We conclude that the presence of such a substance supports the concept of local regulation of bone metabolism.

One of the key points from which bone remodelling can be controlled is at the level of progenitor bone cell proliferation. In the normally coupled processes of resorption and formation, the erosion of bone by osteoclasts is rapidly followed by the proliferation and differentiation of osteoblasts and ultimate replacement of the resorbed bone. That this process of proliferation is finely regulated is indicated by the observation that cell division occurs only in a very limited spatial and temporal area around the resorption site. That is, the production of mature osteoblasts occurs at no greater distance than about 50–100 μm from active osteoclasts and lasts for only as long as is necessary to reform bone in the resorption lacunae. Part of the control over the proliferation of progenitor bone cells in this coupled system has been attributed to a short-ranged diffusable stimulatory factor released by resorbing bone (1). However, in order to achieve the degree of control seen in bone remodelling, one could speculate that a substance which rapidly inhibits cell division might also be present. In order to explore this possibility, we have characterized and utilized serum-free culture systems of embryonic chick long bones and dispersed calvarial cells. The serum-free organ culture system allows the direct recovery of substances released into the medium by the long bones, avoiding the problems encountered with undefined serum supplements, and the serum-free cell culture system allows a rapid quantitation of the effects of these substances on the proliferation of calvarial cells.

Materials and Methods. *Serum-free cell and organ culture.* Isolated embryonic chick calvarial cells were prepared by an adaptation of the methods used for the isolation of fetal rat calvarial bone cells (2). Briefly, the frontal and parietal bones of 17-day embryonic chicks were aseptically dissected and incubated at 37°, with shaking, in minimum essential medium (MEM) containing penicillin (100 units/ml), streptomycin (100 μg/ml), bovine serum albumin (1 mg/ml), and collagenase (2 mg/ml) (Worth-

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ington Type II) for 20 min. The cells released during this time (mostly red blood cells and superficial connective tissue cells) were discarded (3) and the incubation continued for a further 100 min. Collection and washing of the cells were as previously described (3). The cells were then suspended in BGJ_b medium (Fitton–Jackson modified, Gibco), and plated into culture multiwells (16 mm diameter, Falcon) at a concentration of 250 cells/mm^2 of surface area in 1 ml and incubated at 37° in 5% CO_2:95% air for the duration of the experiment.

Tissue from embryonic (19-day) chick liver, skin, and muscle was minced with scissors into pieces no larger than 4 mm^3. The fragments were then incubated in the above described collagenase solution for 120 min and the resulting isolated cells were collected and washed. The cells were then suspended in BGJ_b medium containing 1% fetal bovine serum and plated at 250 cells/mm^2. After 24 hr the cell monolayers were rinsed and the medium was changed to serum-free BGJ_b. Rat calvarial cells were prepared as previously described and cultured directly in serum-free BGJ_b (4). The human osteogenic sarcoma cell line (TE-85) used in these studies was generously donated by Dr. Jørgen Fogh, Sloan Kettering Institute.

The culture of embryonic tibiae and femora was carried out as previously described (5). The medium bathing the bones was collected daily, and stored in glass vials at 4°. This "conditioned medium" as well as other test substances was then added directly to the cell cultures, usually in a volume of 50 μl. The effect of this medium on cell proliferation was assessed as described below. In some experiments dialysis of the conditioned medium was performed in Spectrapor membranes (Spectrum Medical Industries, Los Angeles, Calif.) against fresh BGJ_b medium before analysis.

Cellular proliferation assay. In general, the incorporation of [^{3}H]thymidine ([^{3}H]-dThd) into trichloroacetic acid (TCA)-precipitable material was used as a measure of the proliferation of cells in monolayer culture. Specifically, a rapid assay first reported by Gospodarowicz (6) was validated and routinely used. The assay was performed as follows. [^{3}H]dThd was added directly to the incubation wells 2 hr prior to the end of the culture period (final concentration: 2 μCi/ml). The radioactive media were then removed and the cell layer was briefly rinsed with 1 ml of MEM. A cotton-tipped applicator stick (Q-tip) was then moistened in 12.5% TCA and used to swab the bottom of the culture well. (As judged by microscopic examination all cellular material was removed from the surface of the well.)

The cotton end of the applicator was washed (twice) in 12.5% TCA (10 min each wash) and (once) in 95% ethanol (10 min). For large numbers of samples, this washing procedure was facilitated by placing the sticks, cotton end up, in modelling clay in the corresponding wells of another culture plate. All the applicators can then be washed simultaneously. The cotton tip was then dried, cut from the wooden applicator, and the radioactivity adhering to the cotton fibers was quantified by scintillation spectrometry using 5 ml of Aquasol (New England Nuclear)/sample.

Mechanical scraping with a Teflon spatula and NaOH solubilization of the cell layer was also performed for a comparison to the above method. In these cases the cellular material and 500 μg of added carrier bovine serum albumin (BSA) were precipitated and washed (twice) in cold 12.5% TCA. The precipitated material was then solubilized and the incorporated radioactivity was determined.

A comparison of these three methods under a variety of conditions is presented in Table I. The coefficient of variation (CV) computed for each value indicates that estimation of [^{3}H]dThd incorporation into DNA by NaOH solubilization of the cell monolayer has the greatest degree of precision (smallest CV) with the cotton tip and mechanical scraping methods about equal. The accuracy of the cotton-tip method versus the NaOH and mechanical scraping methods was assessed by plotting the values from the cotton-tip method against each of the other two. Linear regression analysis for the cotton-tip method versus NaOH in-

TABLE I. VALIDATION OF METHODOLOGY

No. of cells plated/well[a]	[^{3}H]Thymidine in DNA (fmole/well)		
	Cotton-tip method	NaOH solublization	Mechanical scraping
5.00×10^4	50.7 ± 5.1 (0.25)	52.9 ± 4.8 (0.22)	46.2 ± 9.2 (0.49)
2.50×10^4	38.2 ± 6.1 (0.39)	42.7 ± 3.1 (0.18)	40.5 ± 8.7 (0.53)
1.25×10^4	12.3 ± 2.1 (0.42)	14.1 ± 2.0 (0.11)	12.5 ± 4.2 (0.82)
6.25×10^3	1.60 ± 0.9 (1.38)	2.21 ± 0.3 (0.33)	1.80 ± 0.8 (1.09)
0	0.01 ± .002 (0.49)	0.01 ± 0.001 (0.24)	0.01 ± 0.002 (0.49)
Control cells[b]	54.6 ± 8.1 (0.36)	57.8 ± 4.1 (0.17)	55.0 ± 6.1 (0.27)
Control cells + 5% fetal bovine serum	294 ± 10 (0.08)	320 ± 8.0 (0.06)	309 ± 12 (0.10)

[a] Cells were plated at the concentration indicated and incubated for 48 hr. [^{3}H]dThd (2 μCi/ml; 56.2 Ci/mmole) was then added for the last 2 hr of incubation. Values are mean ± SEM, $N = 6$. Values in parentheses are the coefficient of variation.

[b] Cells were plated at 5×10^4/well and incubated for 24 hr. 50 μl of FBS was then added to the appropriate wells and incubated for a further 24 hr. [^{3}H]dThd was added for the last 2 hr of culture. Values are mean ± SEM, $N = 8$.

dicates a slope of 1.05 ± 0.03 and a y intercept of 0.63 ± 0.65 and for the cotton-tip method versus mechanical scraping a slope of 0.95 ± 0.05 and a y intercept of 0.63 ± 1.2. Neither the slopes nor the intercepts are statistically ($P < 0.05$) different from 1.0 or 0.0, respectively, for either comparison. The value of this method lies in the fact that it decreases the time for processing large numbers of samples from about 2 days to 2 hr. We, therefore, have employed this technique for all subsequent estimations of thymidine incorporation.

Cell counts in culture were performed by counting the number of cells with a flattened, stellate morphology per microscope field in random areas of a number of culture dishes.

Kinetic studies. The effect of conditioned medium on the uptake of [^{3}H]dThd into a TCA-soluble cellular compartment was also studied. The methods employed for these experiments are adapted from the procedure used to measure ^{45}Ca transport in isolated rat calvarial cells (7). To obtain the initial velocity (V_i) of isotopic thymidine uptake, chick calvarial cells were exposed to [^{3}H]dThd (final concentration: 100 nM) for 1, 2, 5, 10, 30, and 180 min, immediately washed (twice) in a buffer containing 50 μM unlabeled thymidine (4°), and lysed by the addition of 12.5% TCA. Carrier protein (500 μg BSA) was added to each sample and the TCA-precipitable material was removed by centrifugation. An aliquot of the supernatant was then counted as a measure of the unincorporated [^{3}H]dThd taken up by the cells. The V_i of [^{3}H]dThd uptake was obtained from the linear velocity prior to isotopic equilibrium. The effect of a 3-hr exposure of conditioned medium on the maximum velocity (V_{max}) and Michaelis constant (K_m) of thymidine transport was also estimated. This was performed by determining the V_i at different thymidine concentrations (3–100 nM).

Enzyme treatments. Insoluble enzymes bound to agarose or polyacrylamide beads (Sigma) were used for the digestion of substances in the conditioned medium (8, 9). For each enzyme, the reaction was carried out at 37° for 3 hr in an oscillating water bath, with a final enzyme specific activity of 1 unit/ml. The enzyme beads were quantitatively removed by centrifugation at the end of the incubation period and the supernatant was assayed as described above. Fresh BGJ_b medium exposed to the same enzyme conditions was used as a control in the cell culture assay.

Prostaglandin assay. Prostaglandins of the E series in the conditioned medium were assayed by radioimmunoassay as previously described (10).

Data analysis. The moles of [^{3}H]dThd incorporated into DNA were calculated from

the specific activity of isotope (56.2 Ci/mmole). The data are generally expressed as the mean ± 1 SEM. In the cases where the data are expressed as percentage inhibition the mean and standard error were computed from paired samples. Linear regression analysis of the least-squares method was utilized in validation of methodology.

Results. *Serum-free cell culture.* The recovery of cells after collagenase digestion averaged approximately 5×10^6 cells/calvarium. When the cells were plated into culture wells in serum-free BGJ_b medium at a concentration of 250 cells/mm^2 of surface area, they reached confluence in 4–6 days, maintaining the same gross morphology during this time (Fig. 1). All experiments in this report did not exceed 48 hr of culture unless otherwise noted.

Effect of conditioned medium. The medium collected from bones growing in organ culture was assayed for its effect on the proliferation of isolated calvarial cells in monolayer culture. (The protein concentration in this conditioned medium usually was in the range of 10–100 μg/ml.) In these experiments, the calvarial cells were exposed to approximately a 10- or 20-fold dilution of the conditioned medium (i.e., 50 or 100 μl of medium from organ culture added to 1 ml of medium in cell culture). The proliferation rate of the cells was then estimated after different durations of exposure by determining the incorporation of [^{3}H]-dThd into DNA. Treated cells were markedly inhibited by a brief exposure to the conditioned medium, but gradually escaped this inhibition by 24 hr (Fig. 2). The cells escaped inhibition from the 20-fold dilution sooner than from the 10-fold dilution. Addition of conditioned medium (50 μl) to cells which had returned to a control rate of proliferation again inhibited thymidine incorporation after 4 hr. These results demonstrate that an inhibitory substance in the conditioned medium works rapidly to prevent cellular proliferation and suggests that the escape from this inhibition is due to a loss of activity of the IS, rather than to the cells becoming refractory after the initial exposure to IS.

Assay of conditioned medium collected from different days of organ culture indicated that the greatest degree of inhibition was in Day 2 medium.

Proof that this inhibitory activity was not an artifact due to alterations in the accumulation of the [^{3}H]dThd or in the intracellular specific activity of the tracer was obtained from studies of the kinetics of thymidine transport (Figs. 3A, and B). Exposure of the cells to conditioned medium for up to 3 hr had no significant effect on (a) the initial velocity of uptake of [^{3}H]dThd (at 100 nM); (b) the K_m and V_{max} of thymidine transport; or (c) the endogenous pathways for thymidine synthesis as determined by a constant intracellular specific activity for 3 hr. Additional evidence that IS affected cell proliferation was obtained in other experiments in which cell number was estimated after prolonged exposure to IS (three doses of IS given in 36 hr). These results indicated a slight but statistically significant ($P < 0.05$) inhibition in cell division (control cells, 42.7 ± 1.4; IS-treated cells, 37.1 ± 1.5 cells/field, mean ± SEM, $N = 5$).

Figure 4 demonstrates a dose–response experiment in which dilutions of the conditioned medium were tested for their effect on proliferation and protein synthesis. The range of dilution was from 10- to 1000-fold with a half-maximal effect on proliferation at approximately a 300-fold dilution. There was no significant effect of IS on protein synthesis at any dilution. In addition, IS has been shown to inhibit the strong (10-fold) proliferative stimulus of 5% fetal bovine serum (FBS) (serum-free control cells, 49 ± 8; cells and FBS, 550 ± 24; cells and FBS and IS, 210 ± 16 fmole [^{3}H]dThd in DNA/well, mean ± SEM, $N = 8$).

The effect of IS is not mediated through cytotoxicity, as evidenced by the fact that (a) the gross morphology of the cells does not change after exposure to IS (photo not shown); (b) IS has no effect on protein synthesis over a wide dilution range (Fig. 4); and (c) the cells can escape the inhibition of IS at which time they return to a control rate of proliferation (Fig. 2).

Table II demonstrates that IS is not retained during dialysis in tubing with an

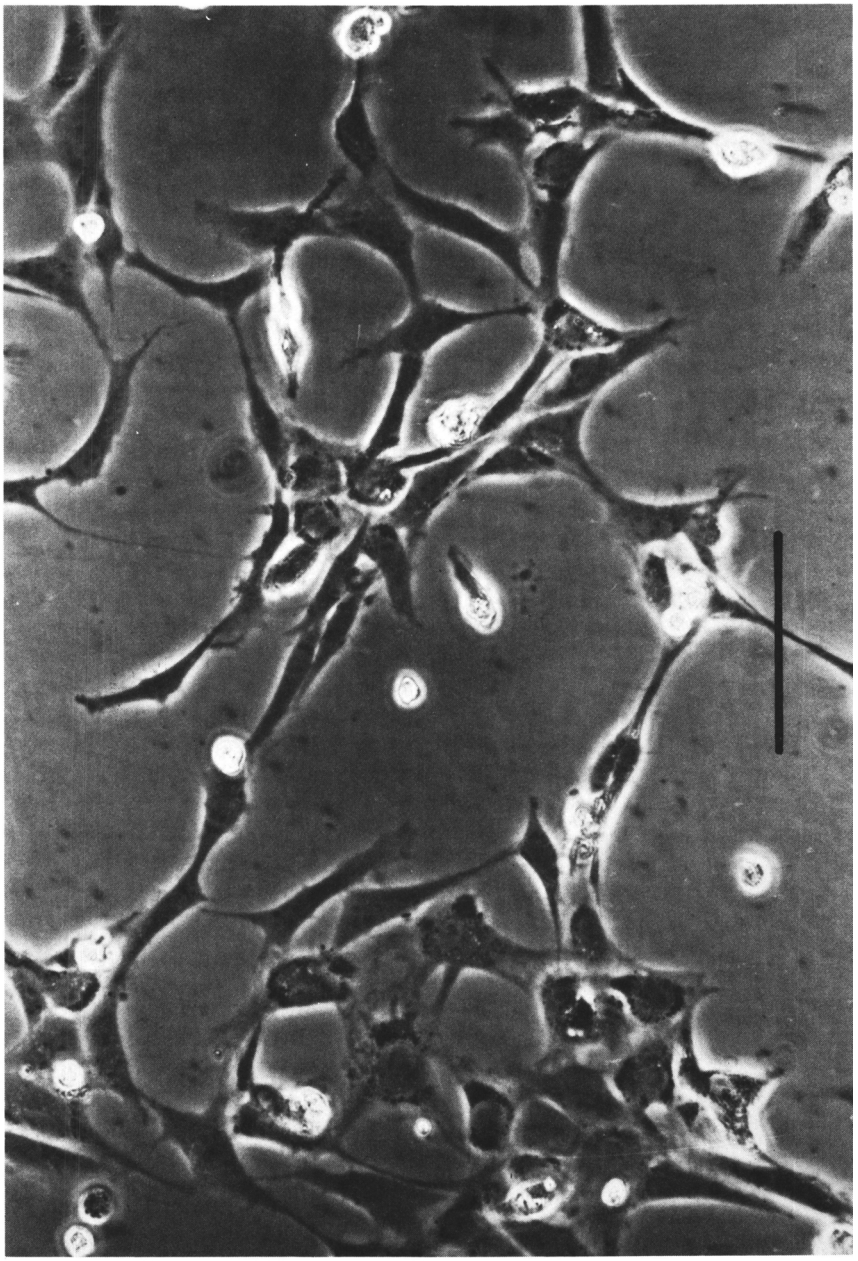

FIG. 1. Morphology of cultured chick calvarial cells. These cells were plated at a density of 250 cells/mm^2 in serum-free BGJ$_b$ medium (Fitton–Jackson modified) and incubated in 95% air:5% CO_2, at 37° for 30 hr. They maintain this same morphology until confluent at approximately 6 days. Bar equals 100 μm.

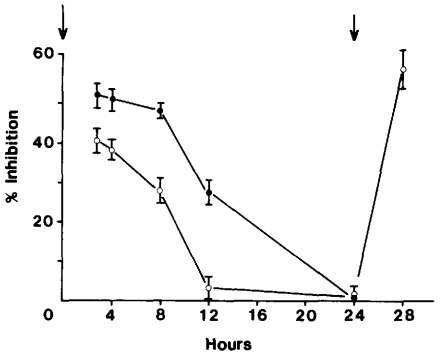

FIG. 2. Inhibition of calvarial cells by IS. Calvarial cells were incubated for 24 hr, at which time conditioned medium was added at a dilution of 1:10 (●) or 1:20 (○) (first arrow). The proliferation of the cells was assayed at the indicated times and expressed as percentage inhibition as compared to untreated cells at the same time. As can be seen the cells appear to escape the inhibition within 12 hr for the 1:20 dilution and 24 hr for the 1:10 dilution. Conditioned medium (1:20) added to cells which had already escaped the inhibition (second arrow) was again inhibitory. $N = 8$ in all cases.

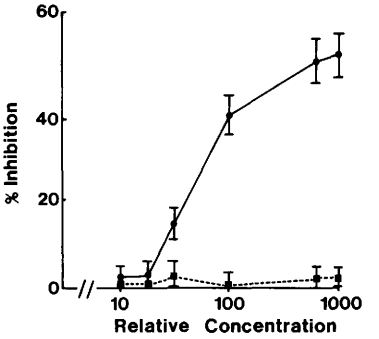

FIG. 4. Dose–Response to IS. Cells were exposed to various dilutions of conditioned medium for 4 hr (from 1:10 to 1:1000). The proliferation rate (as measured by [^{3}H]dThd incorporation) (●) and protein synthesis rate (as measured by [^{3}H]proline incorporation) (■) were both estimated and compared to control-treated cells. As can be seen the proliferation of calvarial cells is markedly inhibited by IS with a half-maximal effect at approximately a 300-fold dilution, whereas there was no effect of IS on protein synthesis at any dilution. $N = 8$ in all cases.

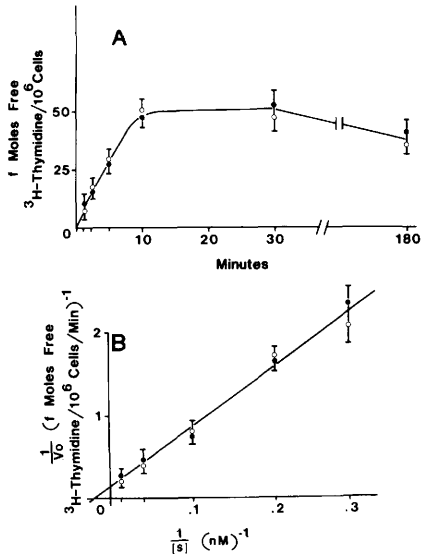

FIG. 3. Kinetic studies of [^{3}H]dThd transport. Panel A: As compared to control cells (●), IS-treated cells (○) showed no significant difference in the accumulation of [^{3}H]dThd from 1 to 180 min (thymidine concentration was 100 nM). Panel B: The kinetic parameters for thymidine transport (K_m and V_{max}) were estimated after a 3-hr exposure of the cells to IS (1:20). Velocity of uptake was measured at 7 min for concentrations ranging from 3.5 to 100 nM. No significant difference was noted between control (●) and IS-treated (○) cells. $N = 4$ in all cases.

exclusion limit of 12,000–14,000 daltons, but that it is retained in tubing with exclusion limits of 6000–8000 and 3500 daltons, respectively. If IS behaves like a globular protein during this dialysis, its molecular weight can be estimated to be 6000 to 14,000 daltons.

The specificity of action of IS is evaluated in Fig. 5. These data indicate that cells derived from bone are particularly sensitive to the inhibitory action of IS, whereas skin fibroblasts and muscle cells are sensitive to a lesser extent and cells derived from liver are not at all sensitive.

It has been previously shown that prostaglandins, especially of the E series, can inhibit the proliferation of cells in monolayer culture (11) and also that bone in organ culture releases PGE_1 and PGE_2 into the surrounding medium (12). In order to rule out the possibility that IS is a prostaglandin, three parameters were measured: (a) the concentration of prostaglandins in the IS-containing medium, (b) the direct effect of exogenously added prostaglandins on cell proliferation, and (c) the extractability of IS in ethyl acetate. As can be seen in Table III, a value of 10^{-11} M was found for PGE_1 in the IS-containing medium. This medium inhibited cell prolif-

TABLE II. DIALYSIS OF IS

	[^{3}H]Thymidine in DNA (fmole/well)[a]		
	Dialysis tubing exclusion limit, daltons		
	12,000–14,000	6000–8000	3500
Retentate from dialysis of fresh medium (1:20 dilution)	55.6 ± 4.1	52.7 ± 3.2	59.4 ± 6.2
Retentate from dialysis of conditioned medium (1:20 dilution)	52.9 ± 4.7	21.1 ± 6.0[b]	25.8 ± 5.3[b]

[a] Mean ± SEM, $N = 8$.
[b] Significantly different from control, $P < 0.001$, by Student's t test.

eration by about 60%. The highest concentration of the exogenously added prostaglandins E_1 and E_2, 10^{-5} M, demonstrated only a slight inhibition. Concentrations of 10^{-6} M and lower were without effect. In addition, after acidification and extraction of the conditioned medium with ethyl acetate (a method used to quantitatively extract prostaglandins), it was not possible to remove any inhibitory activity from the medium, nor to demonstrate inhibitory activity in the extract. Thus, these data support the contention that IS is not a commonly produced prostaglandin which is extractable in ethyl acetate.

Further characterization of IS involved attempts to destroy the inhibitory activity by enzymatic and heat treatment (Table IV). In this series of experiments, the conditioned medium was treated with trypsin, neuraminidase, phospholipase A_2, RNAase, or heat inactivation as described under Materials and Methods. The remaining activity was then assayed in the cell monolayer culture. Inhibitory activity was preserved in all cases. Fresh BGJ_b medium treated with enzymes or heat and assayed in an identical manner provided the controls for this experiment. No statistically significant difference was found between fresh treated medium and fresh medium itself. The control value reported in Table IV is that for fresh medium itself.

Discussion. The data in this paper demonstrate that a substance released into the medium by embryonic chick bone grown in organ culture can rapidly inhibit the proliferation of isolated bone cells *in vitro*.

TABLE III. PROSTAGLANDINS AND IS

Incubation conditions	Prostaglandin concentration[a] (M)	[^{3}H]Thymidine in DNA (fmole/well)
Control		55.1 ± 5.6
IS (1:20 dilution)	10^{-11}	20.8 ± 3.2[b]
Exogenously added PGE_1	10^{-5}	39.8 ± 4.2[c]
	10^{-6}	53.3 ± 2.2
	10^{-9}	57.2 ± 4.8
Exogenously added PGE_2	10^{-5}	44.1 ± 6.2
	10^{-6}	58.7 ± 2.2
	10^{-9}	58.5 ± 3.5
IS extracted with ethyl acetate		18.7 ± 3.9[b]
Ethyl acetate extract		58.1 ± 6.2

[a] Prostaglandin values represent the final concentration in the cell assay system, $N = 8$.
[b] Significantly different from control, $P < 0.001$, by Student's t test.
[c] Significantly different from control, $P < 0.05$, by Student's t test.

TABLE IV. CHARACTERIZATION OF IS

Incubation conditions	[^{3}H]thymidine in DNA (fmole/well)[a]
Control	61.0 ± 4.0
IS (1:20 dilution)	29.5 ± 5.2[b]
IS treated with	
Trypsin	33.4 ± 5.7[b]
Phospholipase A_2	28.4 ± 7.1[b]
Neuraminidase	29.2 ± 5.0[b]
RNAase	32.6 ± 3.6[b]
Heat inactivation (55°, 30 min)	31.7 ± 4.3[b]

[a] Mean ± SEM, $N = 8$.
[b] Significantly different from control, $P < .005$.

The demonstration of such a substance is the first report of a cell proliferation inhibitor obtained from bone and provides further evidence that osseous tissues have an innate capability for self-regulation through the action of locally released and locally acting factors. Other examples of substances produced by bone or bone cells which have been shown to affect bone metabolism include stimulators of bone collagen synthesis (13), and bone formation *in vitro* (1) and *in vivo* (14), chemotactic factors for monocytes (15), and a stimulator of bone cell proliferation (16). We must emphasize, however, that while some or all of these factors potentially could have important physiological relevance, a physiological role has not been established for any of these factors.

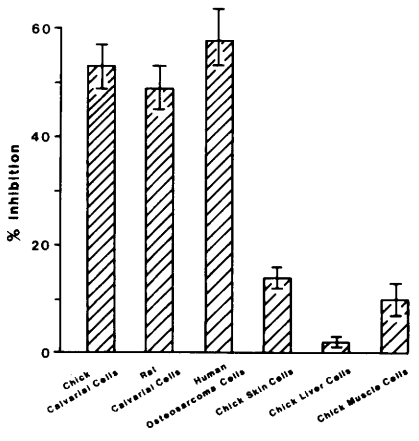

FIG. 5. Specificity of Action of IS. Cells isolated from different tissues and different species were utilized to assay the activity of IS (at a 1:20 dilution). Untreated cells from the same tissues were used as appropriate controls. Cells derived from bone regardless of species were particularly sensitive to the effect of IS, whereas cells from other tissues were considerably less sensitive.

We have shown that a substance recoverable from chick bones growing in organ culture can inhibit cellular proliferation. Our studies demonstrate that IS inhibits the incorporation of a radioactive tracer into DNA and we have interpreted this to be a measure of the inhibition of cell division. This interpretation is supported by experiments indicating that alterations in tracer transport or pool size could not account for the observed effects. Most importantly, under *in vitro* conditions where bone cells are proliferating in culture, IS significantly inhibited the increase in cell number.

While the inhibition of cell proliferation is a real phenomenon, this action could still be the consequence of an *in vitro* artifact and thus have little or no physiological significance. In this regard, the nucleotide, thymidine, has been shown to block cell cycle progression and inhibit DNA synthesis (17), thus raising the possibility that IS is residual thymidine released from the bones in organ culture. However, the reported concentration range for thymidine inhibition is 1–2 m*M* (17) and this high concentration would have been detectable as a change in thymidine specific activity in the kinetic transport measurements. Also, a molecular weight estimation of 6000 to 14,000 for IS and the fact that the cells escape the inhibition of proliferation with time are not consistent with the hypothesis that IS is residual thymidine in the medium.

The present studies disclosed five salient features of this inhibitory substance. (a) IS can be recovered from the culture medium of embryonic growing chick tibiae and femora. (b) IS containing medium can be diluted over 500-fold and still retain significant inhibitory activity. A half-maximal inhibition occurs at a 300-fold dilution of the medium. IS is also effective in inhibiting the strong proliferative stimulus of 5% fetal bovine serum. (c) With regard to the chemical nature of IS, the molecular weight, as estimated by dialysis experiments, is between 6000 and 14,000 daltons. Our results indicate that IS is not a commonly produced prostaglandin, a phospholipid, a polysaccharide containing neuraminic acid residues, RNA, or a large polypeptide containing the exposed amino acids, lysine or arginine. Based on estimated molecular weight, the above features and reports on other inhibitory substances, our working hypothesis is that IS is a small polypeptide. (d) Chick calvarial cells are able to escape the inhibition by IS with time. This escape appears to be due to a degradation of the substance by the cells in culture because further addition of IS to cells which have previously escaped the inhibition is again effective in decreasing proliferation. These data as well as those presented in the Results section, indicate that IS is not cytotoxic, i.e., IS does not have its effect through killing the cells. (e) IS inhibits cells derived from osseous tissues to a much greater extent than cells from other tissues such as liver, muscle, and skin. This substance also appears to be species nonspecific in the sense that cells derived from chick, rat, and human bone are all markedly inhibited.

The best known inhibitors of cell proliferation are chalones. These molecules were originally defined as substances which maintained tissue homeostasis by inhibition of mitosis or some process leading to mitosis. They were stated to be noncytotoxic, tissue specific, species nonspecific, and secreted by the tissue over which they exert their effect (18). IS clearly fills three of these criteria and possibly all four, the only exception being tissue specificity. The fact that chick skin fibroblasts and chick skeletal muscle cells are significantly inhibited, albeit to a lesser degree, argues that IS is not strictly tissue specific. However, an interesting observation bearing on this question is brought out in the data in Fig. 5. Bone cells, like skeletal muscle cells and skin fibroblasts have their origin from the primitive mesenchyme, a mesodermal tissue. It would not be unexpected, therefore, that a small subpopulation of early progenitor cells from muscle and dermis have fundamental similarities with bone progenitor cells in regard to the regulation of their proliferation. As is seen in Fig. 5, the muscle cells and skin fibroblasts, although not to the same extent as the bone cells, are significantly inhibited by IS. In contrast, the liver cells, whose origin is from endoderm, are ontogenetically more distant from bone cells and are virtually unaffected.

One of the most important requirements for the purification of any biological substance is a rapid assay to quantify its effects. In this regard the method described herein should prove valuable for the purification and final characterization of IS.

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