are blue on XG plates because β-galactosidase is present (*7*).

Colorless plaques were picked and tested for ability to transduce a *sep* ts(λ$^+$) lysogenic recipient to temperature insensitivity (Ts$^+$) (*2*). One viable *sep*$^+$ transducing phage, designated λCharon 10-Ec*sep*$^+$1, was further characterized. This hybrid phage (Fig. 1E) was also able to transduce *leuA*, *murE*, and *murF* mutant recipients, and thus carries these three genes as well as the *sep* gene. λCharon 10-Ec*sep*$^+$1 DNA was cleaved by *Eco*RI into three fragments—19.5, 18.2, and 10 kbp in length; and its structure was shown by heteroduplexing the DNA with λ*imm*^{434} DNA (Fig. 1G). A diagram of the λCharon 10-Ec*sep*$^+$1/λ*imm*^{434} molecules is presented in Fig. 1F.

Although the *sep* fragment could have been inserted in either of two orientations (that is, λ*J*, λP′, *leuA sep murE F* λ*R*, or λ*J murF E sep leu* λP′ λ*R*), the actual orientation was the one diagrammed: λ*J* λP′ *leuA sep murE F* λ*R*. This conclusion is based on analysis of two types of heteroduplexes. First, the λCharon 10-Ec*sep*$^+$1/λ*imm*^{434} heteroduplex molecules contained no duplex DNA in the P′ region; if the orientation were λ*J murF E sep leuA* λP′ λ*R*, reannealing would have occurred between the P′ regions of both single strands. Second, heteroduplexes of λCharon 10-Ec*sep*$^+$1 and λ*sep*$^+$82 DNA's were prepared. λ*sep*$^+$82 carries a 10.2-kbp insertion of *E. coli* DNA in which *sep* and *leuA* are in the same orientation as in λ*murF*$^+$121 (Fig. 1C). Heteroduplexes of λ*sep*$^+$82/λCharon 10-Ec*sep*$^+$1 DNA contained a central single-stranded loop in which there was no homology between the *sep-leuA* DNA of λ*sep*$^+$82 and the *leuA-sep* region of λCharon 10-Ec*sep*$^+$1. Therefore, the hybrid phage (λCharon 10-Ec*sep*$^+$1) contains the 18.2-kbp fragment in the orientation which is opposite to that in λ*sep*$^+$82 or λ*murF*$^+$121 (Fig. 1C).

After infection of wild-type *E. coli* by the hybrid phage, penicillin-binding protein 3 was overproduced (Fig. 2). Cells were grown at 30°C and infected with a lysis-defective derivative (*S*am7) of λCharon 10-Ec*sep*$^+$1. Control cells were infected with λ*cI*857*S*am7. Two hours after infection, membrane fractions were isolated and [^{14}C]benzylpenicillin was bound to the membrane proteins (*4*). After treatment with Sarkosyl, the inner membrane proteins were separated by electrophoresis on a polyacrylamide slab gel, and the individual PBP's were assayed by fluorography. Membranes of uninfected cells and cells infected with λ*cI*857 contained normal amounts of the six PBP's, but in cells infected with the hybrid λCharon 10-Ec*sep*$^+$1, PBP-3 was overproduced. The level of PBP-3 in membranes of the hybrid-infected cells was approximately four to five times as high as that in membranes of λ*cI*857-infected or -uninfected cells. Therefore, PBP-3 was overproduced and excess PBP-3 was able to incorporate into inner membrane. The extent of overproduction (that is, total soluble and membrane-bound PBP-3) could not be determined because of the insensitivity of the assay; therefore, the four- to fivefold overproduction is a minimum estimate.

In addition to allowing the amplification of the *sep* gene for the production of PBP-3 for chemical studies of the protein, use of the hybrid phage should facilitate genetic studies of the *sep* region because mutants of *sep* can readily be isolated from λCharon 10-Ec*sep*$^+$1. Although *sep* is an essential gene from *E. coli*, it is not required for growth of λ. Both nonsense and deletion mutations can be expected in the *sep* region. In addition, the hybrid phage should be useful in studying the mechanism of insertion of PBP-3 into the inner membrane. DNA of the hybrid phage could also be used as a probe to measure *sep* messenger RNA synthesis in a study of regulation of *sep* expression.

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Calcification of Differentiating Skeletal Mesenchyme in vitro

Abstract. *Embryonic limb-bud mesenchyme was induced to calcify in culture by the addition of 3 m*M *inorganic phosphate to the medium. Phosphate enhanced calcification of the matrix produced by mesenchymal or fibroblast-like cells, whereas no calcification was evident in areas where cartilage had developed. However, calcification was induced throughout the cell layer by altering the cartilage matrix properties with certain enzymes or by changing the phenotypic expression of the cells with vitamin A.*

The factors that initiate and control the deposition of minerals in animal tissue are not well understood. In general, the production of a calcifiable matrix is required prior to mineral deposition (*1*). In bone, type I collagen is laid down and mineral is then deposited within it (*1*). In cartilage, deposition of mineral is associated with regions of the cartilage from which proteoglycans have been removed (*2*). It has been shown that rat embryonic bone cells (*3*) and embryonic chick limb-bud cells (*4*) are capable of depositing a matrix that calcifies in vitro. However, cellular control of the amount or distribution of calcified matrix in these culture systems has not been demonstrated.

The system we have developed allows control of both the amount and distribution of mineralization. Embryonic chick limb-bud mesenchymal cells were prepared in micro-mass cultures by placing a 20-μl drop containing 4×10^5 cells onto a 35-mm tissue culture dish and allowing the cells to attach themselves before adding the medium [Eagle's minimum essential medium (MEM) supplemented with 10 percent fetal calf serum] (*5*). The mesenchymal cells undergo chondrogenesis (formation of cartilage) within 4 days. Chondrogenesis is characterized by the accumulation of an extracellular matrix consisting primarily of type II [α1 $(II)_3$] collagen (*6*) and proteoglycan aggregates (*7*). The appearance and organization of these matrix components in the limb mesenchyme cultures has been well documented (*8*). The 4-day-old cultures contain a central zone of cartilage nodules that coincide with the region of initial mesenchymal cell attachment and an outer halo of spindle-shaped cells (mes-

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enchymal or fibroblastic in nature) oriented in parallel arrays (Fig. 1A) (*5*). Addition of 3 m*M* inorganic phosphate (KH_2PO_4) to the medium on day 2 (*9*) enhanced calcification of the matrix that was produced by the cell layer in the peripheral zone of the culture by day 6. The presence of mineral in this zone is revealed by chemical analyses (Table 1), energy-dispersive x-ray microanalysis (*10*), von Kossa staining (Fig. 1C), and light and electron microscopy (Fig. 1, E, F, and G). At this time, the presence of mineral in the cartilagenous central zone was negligible compared to the amount present in the peripheral zone. Control cultures that did not contain added phosphate or potassium chloride had no detectable calcified areas when measured by the same methods (Table 1). When 3 m*M* phosphate was added to the medium at the time of initial plating, a marked positive von Kossa stain for calcium phosphate was observed after 4 days in the central zone of the cell layer as well as in the peripheral zone of the culture. The different results of adding phosphate at different times imply that once the mesenchymal cells start to express the chondrogenic phenotype, calcification of the matrix in the central zone is inhibited.

We tried to clarify the relationship of

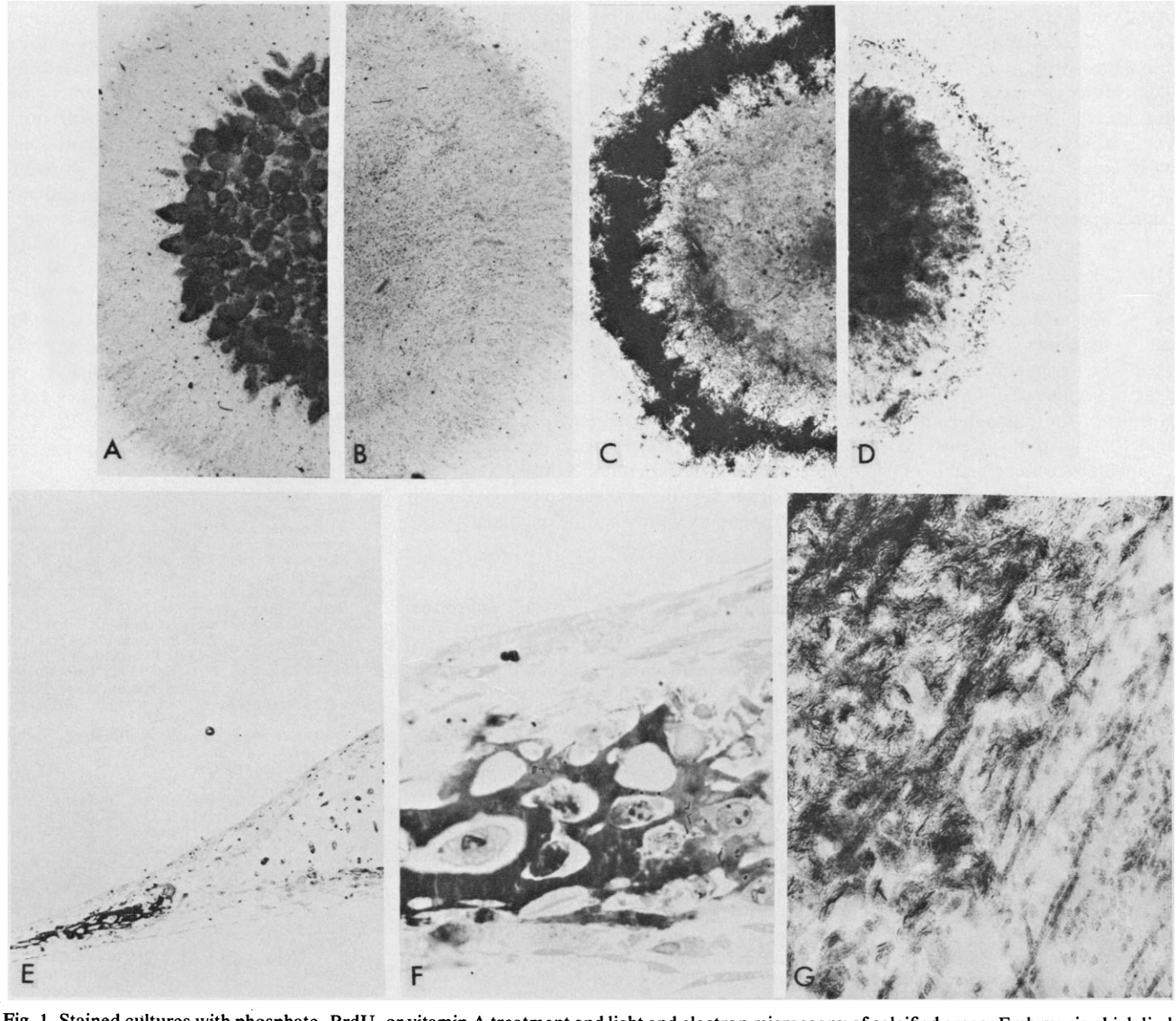

Fig. 1. Stained cultures with phosphate, BrdU, or vitamin A treatment and light and electron microscopy of calcified areas. Embryonic chick limb buds were dissected from stage-24 White Leghorn chick embryos and incubated in 0.1 percent trypsin and 0.1 percent EDTA in calcium- and magnesium-free saline G for 15 minutes. The limb buds were then washed in complete growth medium (Eagle's MEM, 10 percent fetal calf serum, 10 m*M* Hepes buffer, and antibiotics) and mechanically dissociated in fresh complete medium with a vortex mixer. The cell suspension was filtered through two layers of 20-μm mesh Nitex nylon monofilament, and the number of cells was determined. Micro-mass cultures were established by placing a 20-μl drop containing 4×10^5 cells onto a 35-mm tissue culture dish and allowing the cells to attach in a humidified CO_2 incubator (*5*). After 1$^1/_2$ to 2 hours, 2 ml of complete growth medium was added to the cultures. Vitamin A (retinoic acid, 3 μg/ml) or BrdU (33 μM) was added at the time of plating, and the medium was changed every 2 days. On day 2, 10 μl of 0.6*M* KH_2PO_4 was added to the medium of various cultures. The cultures were fixed and stained with alcian blue and metanil yellow on day 6 (*5*). The accumulation of a proteoglycan-rich matrix causes the nodules to stain intensely with alcian blue, whereas the peripheral zone of fibroblast-like cells stains only with metanil yellow. (A) Phosphate-treated control culture stained with alcian blue. Cartilage differentiation is apparent only in the central zone. (B) Culture treated with BrdU or vitamin A. Note the lack of staining by alcian blue. (C) Culture treated with phosphate from day 2 and stained by the von Kossa silver nitrate method (*4*) on day 6. The black reaction product is positive for calcium phosphate mineral deposits. (D) Vitamin A–treated culture with phosphate added, stained by the von Kossa procedure. (E) Light microscopy of a calcified area in the peripheral zone of the phosphate-treated control culture. Deposition of mineral in the matrix surrounding the cells is apparent. (F) Higher magnification of (E). (G) Electron microscopy of a calcified area in the peripheral zone of a control culture.

calcification to the different organic matrices produced by mesenchyme, fibroblasts, and chondrocytes. In one approach, chondrogenesis was inhibited by vitamin A (retinoic acid) (*11*) or by 5-bromo-2′-deoxyuridine (BrdU) (*12*). Both vitamin A and BrdU induced a fibroblastic phenotype (Fig. 1B) characterized by the synthesis of type I collagen and nonaggregating, low-molecular-weight proteoglycans (*10, 11*), but only vitamin A induced a marked calcification of the entire cell layer (Fig. 1D and Table 1). The difference in the actions of vitamin A and BrdU suggests that fibroblast-like cells can produce matrices that differ in their calcification properties.

In a second approach, we tested whether enzymatic degradation of the cartilage proteoglycans may change the matrix properties and allow calcification. Cultures were treated with two kinds of hyaluronidase, *Streptomyces* hyaluronidase (SH), which specifically degrades hyaluronic acid and prevents the aggregation of proteoglycan monomers (*13*), and testicular hyaluronidase (TH), which degrades both the chondroitin sulfate portion of the monomer and hyaluronic acid (*14*). In our systems, SH released ^{35}S-labeled proteoglycans of monomer size only into the medium, whereas the degradation products of TH had a much lower molecular weight (< 3000), being included on a G-25 Sephadex column. Table 1 indicates that SH was significantly more effective in making the proteoglycan matrix calcifiable than TH, suggesting that stimulation of cartilage calcification requires a disaggregation of proteoglycan complexes. There was also a significant increase in alkaline phosphatase activity after treatment with SH or TH (Table 1). Both the SH and TH preparations were analyzed and found to contain no alkaline phosphatase. The mechanism for the increased activity is not well understood.

It is possible that the difference in calcification with SH and TH treatment may result from impurities in the TH preparation. Contaminants in the TH preparation have been shown to inhibit the cartilage phenotype of cultured chondrocytes (*15*). Whether these impurities in the enzyme preparation also influence cartilage calcification remains to be determined. The SH results correlate with those of the studies in vivo and in vitro (*2, 16*), suggesting that calcification of cartilage matrix is initiated by the loss of proteoglycan aggregate structure.

Although calcification can result from cellular degeneration, there is no evidence for this in these cultures. The micrographs show that the calcification occurred entirely within the extracellular matrix in association with collagen fibrils. Furthermore, none of the treatments affect cell viability in this or other chondrocyte culture systems (*11, 12*). It has been reported (*17*) that parathyroid hormone responsive cells (osteoblasts) develop in these cultures, but it remains to be determined whether they contribute to the mineralization.

In previous studies (*9*), high phosphate concentrations (3 m*M* or more) were used to provide a suitable environment for calcification in bone organ culture systems. In this study, we facilitated calcification in a cell culture system by increasing the phosphate concentration. This enabled us to probe the matrix requirements for calcification. By controlling the cellular phenotype and the resulting extracellular matrix, we were able to influence calcification.

Table 1. Staining, ^{45}Ca incorporation, and alkaline phosphate content of control and treated cultures of embryonic chick limb-bud cells. Cultures were prepared as described in the legend to Fig. 1. After 48 hours the medium was replaced with fresh medium containing additional phosphate and the various other drugs. (Control cultures contained no additional phosphate.) We then added BrdU at a final concentration of 33 μM dissolved in MEM and vitamin A at a final concentration of 3 μg/ml. *Streptomyces* hyaluronidase (Calbiochem, B grade) and TH (Sigma, type IV) were added to the medium at concentrations of 1 U/ml and 50 μg/ml, respectively. The medium was changed on day 4 and the different treatments were continued. On day 6, two dishes of each group were fixed and stained separately with alcian blue or von Kossa silver nitrate. In five cultures of each group, ^{45}Ca incorporation and alkaline phosphatase activity were measured. One microcurie of $^{45}CaCl_2$ (specific activity, 11 mCi/mg) was added to each culture. The medium was decanted after 2 hours and the cultures were washed three times with cold 0.15*M* NaCl and 3 m*M* $NaHCO_3$. The cell layer was scraped into 0.5 ml of the same solution, sonicated, and centrifuged at 15,000 rev/min for 20 minutes at 4°C. The supernatant portion was assayed for alkaline phosphatase (E.C. 3.1.3.1), with *p*-nitrophenyl phosphate being used as substrate. One unit of alkaline phosphatase activity was defined as the enzyme activity that liberated 1 mole of *p*-nitrophenol per gram of protein in $^1/_2$ hour at 37°C. The pellet was resuspended in 0.5 ml of 0.1*M* $CaCl_2$ and agitated for 30 minutes. The tubes were centrifuged (3000*g*) and the supernatant portion was aspirated. The pellet was solubilized in 0.5 ml of 0.5*M* HCl for 2 hours at room temperature, and equal portions were used to determine radioactivity. Protein was estimated by the Lowry method. (Staining was judged on a scale of 0 to 4, with 0 representing no stain and 4 the darkest stain.)

	Staining			Chemistry	
		Silver nitrate			
	Alcian blue	Central zone	Peripheral zone	^{45}Ca incorporation (10^{-3} × count/min-mg protein)	Alkaline phosphate (U/g of protein)
Control	4	0	0	1.1 ± 0.1	11.4 ± 0.7
Phosphate	4	0	2	122 ± 13	12.7 ± 1.2
Phosphate + BrdU	1	1	1	178 ± 10	13.7 ± 1.1
Phosphate + vitamin A	0	4	2	956 ± 138	11.9 ± 0.5
Phosphate + SH	1	3	2	491 ± 29	21.2 ± 4.2
Phosphate + TH	1	1	2	184 ± 21	18.5 ± 0.5

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cumulated in the peripheral and central zones, calcium and phosphorus x-ray measurements were conducted on an entire micro-mass culture or on the central and peripheral zones separately. This was done on micro-mass cultures prepared for scanning electron microscopy and coated with carbon. The results showed that 6-day-old cultures treated with phosphate from day 2 accumulated 10 to 15 times more calcium in the peripheral zones and 3 to 4 times more phosphorus. The Ca/P ratio of this mineral was 1.56. There was no significant difference in control cultures without added phosphate between the central and the peripheral zones and the whole cell layer.
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Calmodulin Activation of Adenylate Cyclase in Pancreatic Islets

Abstract. *Pancreatic islets contain calmodulin. The protein binds to a particulate fraction derived from the islets and stimulates adenylate cyclase activity in this subcellular fraction, both phenomena being activated by ionized calcium. A calcium-dependent stimulation of adenylate cyclase by endogenous calmodulin may contribute to the accumulation of adenosine 3',5'-monophosphate evoked by insulin releasing agents in the islet cells.*

Most insulin-releasing agents (secretagogues) examined for such a purpose increase the concentration of adenosine 3',5'-monophosphate (cyclic AMP) in pancreatic islet cells. This applies to secretagogues that are chemically unrelated and do not share a common primary site of action in the B cell such as glucose, α-ketoisocaproate, hypoglycemic sulfonylurea, glucagon, theophylline, barium, and the calcium ionophore A23187 (*1*). In certain cases the accumulation of cyclic AMP is known to be due to a primary activation of adenylate cyclase (for example, by glucagon) or inhibition of phosphodiesterase (for example, by theophylline). In other cases, however, the mechanism leading to cyclic AMP accumulation remains poorly understood. All the above-mentioned secretagogues affect the fluxes and concentration of calcium (or barium) in islet cells (*2*). It is here proposed that the concentration of ionized calcium in islet cells may control the activity of adenylate cyclase by way of the calcium-dependent regulatory protein calmodulin (*3*).

Pancreatic islets were isolated from fed female albino rats (*4*), and the endogenous calmodulin was assayed as described elsewhere (*5*). A group of 2000 islets was homogenized in 0.4 ml of a solution of ethylenebis(oxyethylenenitrilo) tetraacetic acid (EGTA; 0.1 m*M*), and portions (1, 5, and 25 μl) of this homogenate were used in the assay. For the measurement of adenylate cyclase activity, the isolated islets were homogenized in tris-HCl buffer (25 m*M*, *p*H 7.6) containing $MgCl_2$ (5.0 m*M*), EGTA (1.5 m*M*), and dithiothreitol (3.0 m*M*) in a ratio of 100 islets to 0.2 ml of buffer. After a first centrifugation for 1 minute at 100*g* to remove cell debris and intact cells, the homogenate was either used as such or centrifuged for 20 minutes at 27,000*g* and 4°C. The pellet was resuspended and centrifuged again two to three times in the same volume of the same tris-HCl buffer. All experiments were conducted during 20 minutes of incubation at 37°C in a final volume of 75 μl (*6*). The reaction mixture contained (final concentration) tris-HCl (25 m*M*, *p*H 7.6), $MgCl_2$ (5.0 m*M*), EGTA (0.3 m*M*), 3-isobutyl-1-methylxanthine (0.5 m*M*), dithiothreitol (0.6 m*M*), bovine albumin (0.5 mg/ml; bovine albumin, fraction V, Sigma), ^{32}P-labeled adenosine triphosphate ([α-^{32}P]ATP) (13 μ*M*; 20 μCi/ml), an ATP-regenerating system consisting of creatine phosphate (20 m*M*) and creatine kinase (0.8 mg/ml; E.C. 2.7.3.2), and 15 μl of either the islet homogenate (4.6 ± 0.1 μg of protein) or particulate fraction derived from it (1.9 ± 0.1 μg of protein). The reaction mixture also contained, as required, $CaCl_2$ (up to 5.0 m*M*), guanosine triphosphate (GTP; 10.0 μ*M*), unlabeled calmodulin (up to 7 μ*M*), and NaF (10.0 m*M*). The enzymatic reaction was initiated by addition of the islet homogenate or particulate fraction, and terminated as described in (*7*). The separation of cyclic AMP from ATP was achieved by the procedure of Salomon *et al.* (*8*) with the use of Dowex 50 W-X4 and neutral alumina columns. For the measurement of ^{125}I-labeled calmodulin binding, the particulate fraction was added (time zero) to the same reaction mixture as that used in the assay of adenylate cyclase activity, except for the absence of the tracer amount of [α-^{32}P]ATP and presence of ^{125}I-labeled calmodulin (16 n*M*). After 20 minutes of incubation at 37°C, the binding reaction was terminated as described elsewhere. The separation of bound from free ^{125}I-labeled calmodulin was performed by immediate filtration of the diluted reaction mixture through EHWP Millipore filters (*9*). The labeled and unlabeled calmodulin were prepared from bovine pancreas as described (*5*, *9*). All results are expressed per milligram protein and presented as the mean (± standard error). Protein was determined by the method of Lowry *et al.* (*10*), bovine albumin being used as a standard.

The islets were found to contain calmodulin at a concentration of 0.13 pmole per islet, that is, about 0.2 pmole per microgram of protein or, assuming equal distribution in the intracellular water space (2 to 3 nl per islet), approximately 50 μ*M*. The latter concentration is somewhat higher than that reported (1 to 20 μ*M*) in other tissues (*3*).

The binding of ^{125}I-labeled calmodulin (16 n*M*) to a membrane-rich particulate fraction prepared from the islet homogenate was measured in the presence and absence of unlabeled calmodulin (7 μ*M*) to correct for nonspecific binding (*11*). The influence of the concentration of ionized calcium on the specific binding of ^{125}I-labeled calmodulin was investigated by adding $CaCl_2$ (0.05 to 4.0 m*M*) to the reaction mixture containing 0.3 m*M* EGTA. Relatively little labeled calmodulin was bound when small amounts of $CaCl_2$ were added (0.1 m*M* or less). A tenfold increase in binding was observed when the amount of added $CaCl_2$ was raised to 0.4 m*M* (Fig. 1A). Higher concentrations of $CaCl_2$ did not further enhance the binding of labeled calmodulin (*12*).

The effect of bovine pancreatic calmodulin (7 μ*M*) on the activity of adenylate cyclase was judged from paired measurements performed at different calcium concentrations in the absence or presence of the protein (*13*). To minimize the contamination by endogenous calmodulin and GTP, we performed these

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