3), as well as the free enkephalins, have such amino terminal sequences and interact with the opiate receptor. It remains to be seen, therefore, whether peptides other than the free [Met]- and [Leu]enkephalins are of physiologic importance among all those found in the adrenal gland. On a molar basis the free enkephalins represent no more than 9 percent of the total compounds containing enkephalin sequences (Table 1). On a weight basis, the enkephalins represent less than 0.4 percent of the total. Conceivably, some of the larger peptides and proteins may provide additional specificity for cell recognition, confer additional stability in vivo, or even possess different types of biological activity.

The finding of the sequences of three different hormones in pro-opiocortin was rather surprising at the time (*14*). It is of interest that the putative enkephalin precursors also contain more than one active peptide sequence, [Met]enkephalin and [Leu]enkephalin. What is most unusual about the larger peptides and proteins is that they contain multiple copies of a single sequence, [Met]enkephalin. The full significance of this multiplicity of the [Met]enkephalin sequence in the large peptides is not clear. It does, however, explain the ratio of [Met]enkephalin to [Leu]enkephalin of about 5 to 1 up to 7 to 1 reported by others (*16*), and suggests that this ratio is already encoded in nuclear DNA. Another important conclusion is that a common precursor to [Met]enkephalin and [Leu]enkephalin makes unlikely the proposed occurrence of separate neurons for the two enkephalins (*17*).

A 50,000-dalton enkephalin-containing protein has also been found in the striatum and intestine (*2*), and the heptapeptide, [Met]enkephalin-Arg^6-Phe^7, and other extended enkephalins found in adrenal medulla are also present in striatum (*6*). Thus, the biosynthetic pathway for enkephalins suggested by these studies on the adrenal medulla may be universal for all enkephalin-containing tissues.

RANDOLPH V. LEWIS, ALVIN S. STERN
SADAO KIMURA*, JEAN ROSSIER†
STANLEY STEIN, SIDNEY UDENFRIEND
Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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1. Abbreviations: Met, methionine; Tyr, tyrosine; Gly, glycine; Phe, phenylalanine; Leu, leucine; Arg, arginine.
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* Present address: Division of Biochemistry, Tsukuba University School of Medicine, Sakura, Niihari, Ibaraki, 300-31, Japan.
† Present address: CNRS Physiologie Nerveuse, 91-90 Gif-sur-Yvette, France.

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Sodium-Calcium Exchange Activity Generates a Current in Cardiac Membrane Vesicles

Abstract. *Sarcolemmal membrane vesicles isolated from canine ventricular tissue accumulate calcium through the sodium-calcium exchange system when an outwardly directed sodium gradient is generated across the vesicle membrane. Moreover, calcium uptake under these conditions is accompanied by the transient accumulation of the lipophilic cation tetraphenylphosphonium. Since the distribution of tetraphenylphosphonium across biological membranes reflects the magnitude and direction of transmembrane potential differences and the characteristics of the transient accumulation of this cation closely resemble those of sodium-calcium exchange activity, it is concluded that a membrane potential, interior negative, is produced during calcium accumulation through the exchange system. Thus, the operation of the sodium-calcium exchange system generates a current in cardiac membrane vesicles, suggesting that three or more sodium ions exchange for each calcium ion.*

Calcium ions traverse the myocardial cell membrane in both directions during each cycle of contraction and relaxation in the heart. These Ca^{2+} movements appear to play a crucial role in excitation-contraction coupling in cardiac muscle since the removal of extracellular Ca^{2+} leads to an abrupt loss of contractile activity (*1*, *2*). An important mechanism for the transfer of Ca^{2+} across cell membranes in excitable tissues is Na^+-Ca^{2+} exchange, a carrier-mediated transport process that couples the movement of Ca^{2+} in one direction to the movement of Na^+ in the other (*3*-*5*). The precise role of Na^+-Ca^{2+} exchange in cardiac physiology is uncertain. Reuter and co-workers (*3*, *6*) proposed that its primary function is removal of Ca^{2+} from the cell, utilizing the energy of the inwardly directed Na^+ gradient for this purpose. In contrast, Langer *et al.* (*7*) suggested that it brings Ca^{2+} into the cell with each contraction in response to a depolarization-induced elevation in intracellular Na^+. It has also been suggested that Na^+-Ca^{2+} exchange mediates the inotropic effects of cardiac glycoside administration and changes in stimulation frequency (*4*, *8*).

The stoichiometry of the cardiac Na^+-

Table 1. Uptake of TPP^+ and calcium by cardiac membrane vesicles treated with valinomycin or carbonyl cyanide *m*-chlorophenylhydrazone.

Treatment*	TPP^+ uptake† (nmole per mg of protein)		Ca^{2+} uptake† (nmole per mg of protein)
	EGTA	Ca^{2+}	
Control	0.58 ± 0.06	1.64 ± 0.07	35.2 ± 2.4
Valinomycin	0.37 ± 0.04	0.40 ± 0.05	61.8 ± 1.1
Carbonyl cyanide *m*-chlorophenylhydrazone	0.42 ± 0.10	0.60 ± 0.04	51.6 ± 4.4

*Vesicles were treated with $8.3 \times 10^{-6} M$ valinomycin (4 nmole per milligram of protein) or $3.3 \times 10^{-6} M$ carbonyl cyanide *m*-chlorophenylhydrazone (2 nmole per milligram of protein) and then assayed for TPP^+ and Ca^{2+} uptake as described in the legend of Fig. 1. †Values are the means ± standard errors of triplicate determinations. For both the TPP^+ and Ca^{2+} uptake studies, Ca^{2+} was present at a concentration of 50 μM. Uptake was measured over a 10-second interval in each case.

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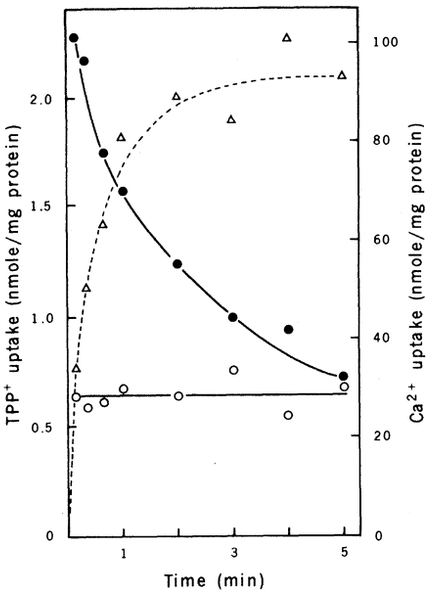

Fig. 1. Uptake by cardiac membrane vesicles of ^{3}H-labeled TPP^+ measured in the (●) presence or (○) absence of 50 μM $CaCl_2$, and of (△) $^{45}Ca^{2+}$ measured in the presence of 50 μM $^{45}CaCl_2$. Sarcolemmal membrane vesicles were prepared from canine ventricular tissue by a combination of differential and sucrose density gradient centrifugation (*23*). Vesicles were loaded internally with NaCl by incubation for 18 hours at 0°C in 160 mM NaCl, 20 mM Mops-tris (pH 7.4). To assay TPP^+ uptake, 0.5-μl portions of the Na^+-loaded vesicles (5 to 10 mg of protein per milliliter) were added to tubes containing 15 μl of 160 mM KCl, 20 mM Mops-tris (pH 7.4) and 33 μM [^{3}H]TPP^+ (2.5 Ci/mmole; bromide salt) with (●) 50 μM $CaCl_2$ or (○) 0.1 mM EGTA present. Uptake was terminated by diluting the contents of the tube with 5 ml of ice-cold 200 mM KCl, 5 mM Mops-tris (pH 7.4). The vesicles were immediately collected on Whatman GF/A glass fiber filters and washed twice with 5 ml of 200 mM KCl, 5 mM Mops-tris. Dilution, filtration, and washing were accomplished within 8 seconds; the half-time of TPP^+ loss in the termination medium at 0°C was ~ 15 seconds. To measure Ca^{2+} uptake, 0.5-μl portions of the Na^+-loaded vesicles were diluted into 160 mM KCl, 20 mM Mops-tris (pH 7.4) containing 50 μM $^{45}CaCl_2$ (Amersham; 0.8 Ci/mmole); uptake was terminated by the filtration procedure described above. In the presence of 33 μM TPP^+ the initial rate and extent of Ca^{2+} accumulation were ~ 80 percent of the values in the absence of TPP^+ (data not shown).

Ca^{2+} exchange system is a subject of debate (*9*). Electroneutral exchange of two Na^+ per Ca^{2+} is indicated by the finding that Na^+-dependent Ca^{2+} fluxes in guinea pig atria are a function of the square of the Na^+ concentrations on either side of the membrane and are not affected by changes in the membrane potential (*3, 6*). Electrogenic exchange of at least three Na^+ per Ca^{2+} is suggested by studies of the dependence of contractile tension on the external Na^+ concentration and the membrane potential in frog atrial tissue (*1, 10*). In nervous tissues Na^+-Ca^{2+} exchange appears to be an electrogenic process (*11*). An electrogenic exchange system could have several advantages over an electroneutral process with respect to its possible physiological functions in the heart. A three-for-one exchange system would remove Ca^{2+} from the cell much more effectively than a two-for-one process (*9, 12*). Moreover, the activity of an electrogenic process would respond to changes in the membrane potential, bringing Ca^{2+} into the cell on depolarization and removing it from the cell on repolarization (*9*). Finally, an electrogenic exchange system might also play an important role in the genesis of membrane currents during the cardiac action potential (*9*).

We have shown that a subcellular preparation of cardiac membrane vesicles accumulates Ca^{2+} through the Na^+-Ca^{2+} exchange system when an outwardly directed Na^+ gradient is generated across the vesicle membrane (*13*). Pitts (*14*) confirmed these results and reported that the stoichiometry for Na^+-Ca^{2+} exchange in the vesicle system is three Na^+ per Ca^{2+}. Pitts's conclusion was based on direct measurements of Na^+ and Ca^{2+} fluxes. The Na^+ flux measurements, however, are likely to be complicated by contributions from Na^+-Na^+ exchange (*15*). Moreover, as pointed out by Pitts (*14*), a three-to-one stoichiometry does not necessarily imply that Na^+-Ca^{2+} exchange is electrogenic, since other ions might participate in the exchange process. In this report, we demonstrate that Na^+-Ca^{2+} exchange activity alters the transmembrane potential in sarcolemmal membrane vesicles derived from canine ventricular tissue. The results provide strong evidence that Na^+-Ca^{2+} exchange is electrogenic in the vesicle system.

We detected the formation of a membrane potential during Na^+-Ca^{2+} exchange activity by monitoring the uptake of a lipid-soluble cation, tetraphenylphosphonium (TPP^+) (*16*). Lipid-soluble ions such as TPP^+ equilibrate rapidly across biological membranes; thus their distribution across the membrane reflects the magnitude and direction of a transmembrane potential. These ions have been used to measure membrane potentials in systems as diverse as mitochondria (*17*), bacterial membrane vesicles (*18*), and intact mammalian cells (*19*). The data in Fig. 1 show the time course of TPP^+ accumulation when sarcolemmal membrane vesicles loaded internally with NaCl (Na_i^+) were diluted 30-fold into a KCl medium containing either 50 μM $CaCl_2$ or the Ca^{2+} chelating agent [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (EGTA). In the absence of Ca^{2+}, TPP^+ was taken up to a constant level of approximately 0.6 nmole per milligram of protein (*20*). When 50 μM $CaCl_2$ was included in the dilution medium, the vesicles accumulated more than three times this amount of TPP^+ within 10 seconds (*21*); the level of accumulated TPP^+ subsequently declined toward the value seen without Ca^{2+} (Fig. 1). We attribute the enhanced uptake of TPP^+ in the presence of Ca^{2+} to the development of a membrane potential, negative inside, produced by the

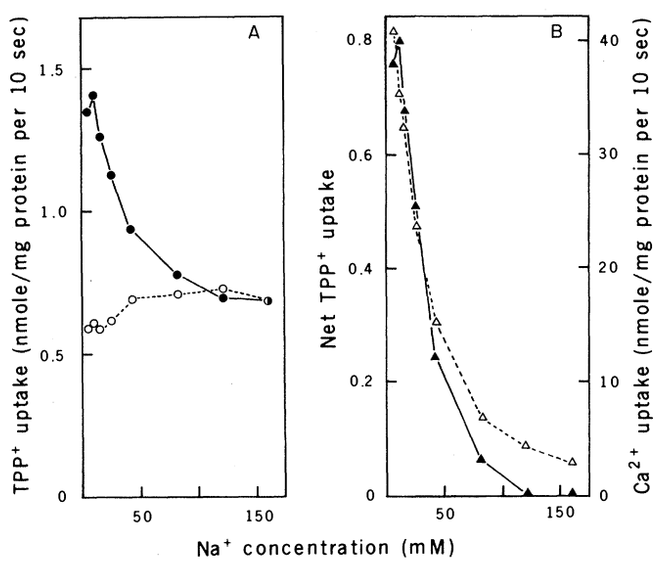

Fig. 2. (A) Uptake of ^{3}H-labeled TPP^+ in the presence of varying concentrations of Na^+. The uptake was assayed in the (●) presence or (○) absence of 100 μM $CaCl_2$ as described in the legend of Fig. 1, except that the dilution media contained admixtures of 160 mM NaCl and 160 mM KCl to give the final Na^+ concentrations shown. The values given are the means of 10-second measurements in triplicate. (B) Effects of Na^+ on (▲) net TPP^+ uptake and (△) Ca^{2+} uptake. The $^{45}Ca^{2+}$ uptake (10 seconds) was measured in triplicate as described in the legend of Fig. 1, except that the concentration of $^{45}CaCl_2$ was 100 μM. Net TPP^+ uptake was calculated as the difference between the values shown in (A) for TPP^+ uptake in the presence and absence of 100 μM $CaCl_2$.

operation of the Na^+-Ca^{2+} exchange system. The falloff in accumulated TPP^+ presumably reflects the decline in the rate of net Ca^{2+} uptake under these conditions. As shown by the slope of the dashed line in Fig. 1, the rate of Ca^{2+} uptake was maximal during the first 10 seconds after the dilution step and gradually declined thereafter as Ca^{2+} uptake approached a steady-state level of accumulation. The current generated by the Na^+-Ca^{2+} exchange system, and hence the change in membrane potential, would be expected to decay toward zero as the steady state is approached. An additional factor leading to the falloff in TPP^+ accumulation is the decline in the driving force for Na^+-Ca^{2+} exchange as the Na^+ gradient dissipates.

An alternative interpretation of the data in Fig. 1 is that Ca^{2+} might activate a channel that allows Na^+ to diffuse out of the vesicle, thus creating a negative diffusion potential. However, we feel that this is unlikely because tetrodotoxin (0.16 m*M*) and D-600 (0.1 m*M*), agents that antagonize known Na^+ currents in cardiac tissue (*22*), have no effect, either alone or in combination, on the enhancement of TPP^+ uptake by Ca^{2+}. Moreover, the characteristics of the Ca^{2+}-induced stimulation of TPP^+ uptake closely resemble those of Na^+-Ca^{2+} exchange activity, as illustrated by the following observations. First, a Ca^{2+}-dependent enhancement of TPP^+ uptake is not observed when Li^+-loaded vesicles are used instead of Na^+-loaded vesicles. Moreover, K^+-loaded vesicles fail to show a Ca^{2+}-induced stimulation of TPP^+ uptake when diluted 30-fold into a choline chloride medium. These results are consistent with the properties of Na^+-Ca^{2+} exchange, since neither Li^+ nor K^+ will substitute for Na^+ in the cardiac vesicle exchange system (*13*). Second, $LaCl_3$ (1 m*M*), an inhibitor of Na^+-Ca^{2+} exchange activity in cardiac vesicles (*13*), abolishes the enhancement of TPP^+ accumulation by Ca^{2+}. Third, Na_i^+-dependent Ca^{2+} uptake and the Ca^{2+}-dependent enhancement of TPP^+ accumulation show a similar dependence on temperature; the activation energy for both processes is 18 kcal/mole over the range 10° to 37°C. Fourth, the stimulation of TPP^+ uptake by Ca^{2+} approaches an upper limit as the external Ca^{2+} concentration is increased; half-maximal enhancement is observed at 8 μM Ca^{2+}, a value that agrees with the Michaelis constant, K_m, of 20 μM for Na_i^+-dependent Ca^{2+} uptake (*13*, *14*). Finally, Ca^{2+}-dependent TPP^+ uptake and Na_i^+-dependent Ca^{2+} uptake are inhibited by external Na^+ over similar concentration ranges. In Fig. 2A, TPP^+ accumulation with and without Ca^{2+} is plotted as a function of the external Na^+ concentration. In the absence of Ca^{2+}, Na^+ has little if any influence on TPP^+ accumulation. In the presence of 100 μM Ca^{2+}, increasing concentrations of Na^+ progressively reduce TPP^+ uptake until values equal to those observed in the absence of Ca^{2+} are attained. In Fig. 2B, the magnitude of the Ca^{2+}-dependent stimulation in TPP^+ uptake is shown along with the rate of Ca^{2+} uptake at each Na^+ concentration. A striking parallelism is observed between the effects of external Na^+ on Ca^{2+} uptake and on the Ca^{2+}-dependent enhancement of TPP^+ accumulation. These results strongly suggest that the membrane potential generated in the presence of Ca^{2+} reflects the activity of the Na^+-Ca^{2+} exchange system.

An increase in the conductivity of the vesicle membrane should abolish the potential generated by Na^+-Ca^{2+} exchange without inhibiting exchange activity itself. This prediction is borne out by the data in Table 1, which show that Ca^{2+} fails to enhance TPP^+ uptake in vesicles treated with either the K^+-specific ionophore valinomycin or the H^+-specific ionophore carbonyl cyanide *m*-chlorophenylhydrazone. Moreover, both ionophores markedly stimulate the initial rate of Ca^{2+} accumulation by the vesicles. The latter result suggests that Na^+-Ca^{2+} exchange activity becomes self-limiting because of the buildup of charge across the vesicle membrane; the ionophores presumably allow the charge to dissipate and thereby enhance the rate of Na^+-Ca^{2+} exchange.

In summary, our data demonstrate that Na^+-Ca^{2+} exchange activity generates a membrane potential in a subcellular cardiac vesicle preparation. The results thus provide direct evidence that the Na^+-Ca^{2+} exchange system in canine cardiac tissue is electrogenic.

JOHN P. REEVES
JOHN L. SUTKO
Departments of Physiology, Internal Medicine, and *Pharmacology, University of Texas Health Science Center, Dallas 75235*

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$$[Ca^{2+}]_i = [Ca^{2+}]_o \left(\frac{[Na^+]_i}{[Na^+]_o}\right)^3 \exp\left(\frac{E_m F}{RT}\right)$$
where E_m is the membrane potential and F, R, and T are Faraday's constant, the gas constant, and absolute temperature, respectively. Substituting $E_m = -70$ mV and the concentrations given earlier into the expression above, we obtain $[Ca^{2+}]_i$ = 3 n*M*. Thus, an electrogenic three-for-one exchange system removes Ca^{2+} from the cell more effectively than an electroneutral two-for-one process.
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21. A threefold increase in TPP^+ accumulation would be equivalent to a change in the magnitude of the membrane potential of approximately 30 mV. This probably underestimates the actual change in membrane potential since some of the accumulated TPP^+ is lost during the filtration and washing procedure used to harvest the vesicles (Fig. 1).
22. Tetrodotoxin blocks the fast inward Na^+ current in cardiac muscle; D-600 antagonizes the slow inward current. The latter current appears to be carried by both Ca^{2+} and Na^+ [E. Carmeliet and J. Vereecke, in *Handbook of Physiology*, R. M. Berne, N. Sperelakis, S. R. Geiger, Eds. (American Physiological Society, Bethesda, Md., 1979), sect. 2, vol. 1, p. 269].
23. Canine ventricular tissue was lightly homogenized in a Waring blendor (low speed, three 5-second bursts) in 0.3*M* sucrose, 0.5 m*M* (ethylenedinitrilo)tetraacetic acid (EDTA) buffered with 10 m*M* *N*-morpholinopropanesulfonic acid adjusted to *p*H 7.0 with tris(hydroxymethyl)-

methylamine (Mops-tris). After centrifugation for 10 minutes at 600*g*, the pellet was washed three times with fresh sucrose-EDTA buffer and finally homogenized vigorously with a Polytron Pt-20 homogenizer. In some cases the homogenate was then incubated with deoxyribonuclease I (10 μg/ml) (K. D. Philipson and D. M. Bers, personal communication) for 30 minutes at 22°C. After centrifugation at 12,000*g* for 10 minutes, the supernatant was layered over 0.6*M* sucrose, 0.5 m*M* EDTA, 10 m*M* Mops-tris (*p*H 7.0) and centrifuged at 113,000*g* for 90 minutes in a Beckman SW 27 rotor. The vesicles were collected at the 0.6*M*/0.3*M* sucrose interface, washed, and resuspended in 160 m*M* NaCl, 20 m*M* Mops-tris (*p*H 7.4). The vesicles were enriched more than 30-fold over the crude homogenate in $(Na^+ + K^+)$-activated adenosinetriphosphatase activity (40 to 60 μmole of adenosine triphosphate hydrolyzed per milligram of protein per hour), a well-characterized sarcolemmal marker. The Na^+-Ca^{2+} exchange activity has been shown to be a property of the sarcolemmal membranes within the vesicle preparation (*13*, *14*).

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Efficient Metal-Ion Catalyzed Template-Directed Oligonucleotide Synthesis

Abstract. *The Pb^{2+} and Zn^{2+} ions are efficient catalysts for the polycytidylic acid–directed polymerization of an activated guanylic acid derivative, guanosine 5′-phosphorimidazolide. The products include oligomers of 30 to 40 units in length. The nucleotide residues are predominantly 2′–5′ linked when Pb^{2+} is the catalyst, and predominantly 3′–5′ linked in the presence of Zn^{2+}. The significance of these results in the context of the prebiotic evolution of RNA polymerase is discussed.*

The DNA and RNA polymerases and DNA ligase differ from most other enzymes in that their specificity is dependent on the interaction of their substrates with a complementary template, as well as on the more normal interaction of substrate with enzyme. Naylor and Gilham were the first to use an analogous template principle to bring about a chemical condensation between oligonucleotides (*1*). They showed that poly(dA) (*2*) facilitates the condensation of two hexathymidylic acid molecules to form dodecathymidylic acid in aqueous solution. This reaction is in many ways analogous to that performed by a DNA ligase.

Organized helical structures are formed between polyuridylic acid and monomeric adenosine derivatives or between polycytidylic acid and monomeric guanosine derivatives (*3*). These structures, although often triple-helical, have much in common with double-stranded DNA and RNA helices. This suggested that it should be possible to carry out template-directed nonenzymatic reactions in which activated monomeric purine derivatives condense together to give oligonucleotides. Polymerase reactions should be susceptible to modeling more easily than most enzymatic oligomerizations, since part of the normal function of the enzyme is taken over by the template.

We have described the template-directed condensation reactions of adenosine derivatives (*4*, *5*) and shown that poly(U) directs the synthesis of oligoadenylic acids—at least up to the octamer—from a suitable adenosine derivative, the 5′-phosphorimidazolide, ImpA. The product formed in this nonenzymatic reaction is predominantly 2′-5′ linked (*5*). We have also shown that the Pb^{2+} ion catalyzes the formation of the longer oligomers, and increases the proportion of the natural 3′-5′ linkage in the products (*6*).

Until recently, we were unable to study the corresponding reactions of the 5′-phosphorimidazolide of guanosine (ImpG) in comparable detail, owing to the difficulty of separating oligo(G)'s in paper chromatographic systems. Our modification of an RPC-5 column (*7*) has permitted a rapid resolution of oligomers at least up to the 40-nucleotide polymer and has allowed separation of linkage isomers. This has enabled us to extend our work considerably. We now report a remarkable catalytic effect of Zn^{2+} and Pb^{2+} on the efficiency and stereoselectivity of the template-directed oligomerization of ImpG.

Polycytidylic acid was prepared by a modification of the method of Steiner and Beers (*8*); unlabeled and ^{14}C-labeled ImpG were prepared by modifications of the procedures of Lohrmann and Orgel (*9*). $(Gp)_n$ markers were prepared by partial alkaline hydrolysis of poly(G). All other reagents were analytical grade.

Paper chromatography was carried out on Whatman 3MM paper, with a mixture

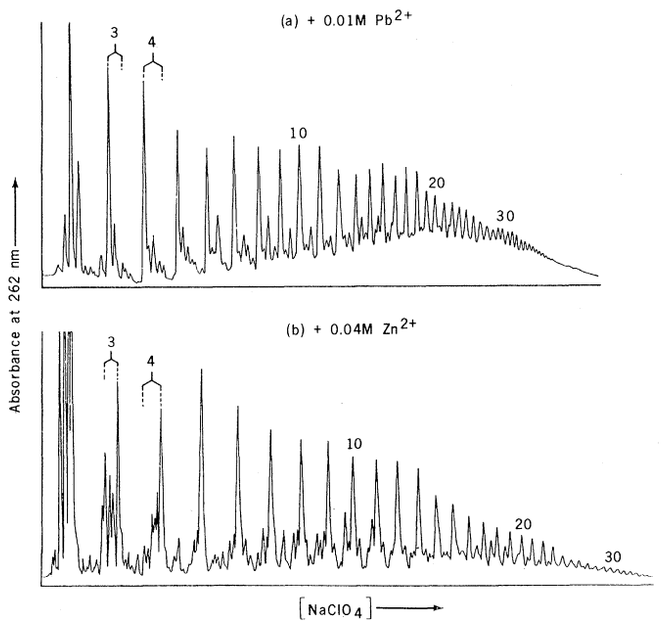

Fig. 1. Elution profiles of products from the template-directed self-condensation of ImpG in the presence of (a) 0.01*M* Pb^{2+} or (b) 0.04*M* Zn^{2+}. The positions of the major peaks of the 10-, 20-, and 30-nucleotide oligomers are indicated. The positions of all 2′-5′- and all 3′-5′-linked isomers of $(pG)_3$ and $(pG)_4$ are also indicated. The reaction conditions were as follows: 0.02*M* ImpG* (0.25 mCi/mmole), 0.04*M* poly(C), 0.4*M* $NaNO_3$, 0.5*M* $Mg(NO_3)_2$, 0.4*M* 2,6-lutidine buffer, *p*H 7.0. After the reaction mixture was held for 12 days at 0°C, excess EDTA was added, and the *p*H was adjusted to 7.9 with tris buffer. Pancreatic ribonuclease [0.25 mg per micromole of poly(C)] was added, and the solution was incubated at 37°C for 8 hours. Material on chromatograms (a) and (b) accounts for 86.2 and 74.6 percent of the products, respectively. The remaining product, pG, eluted with the void volume (not shown).

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