Proc. Nat. Acad. Sci. USA
Vol. 69, No. 2, pp. 480–484, February 1972

Direct Evidence for a Prostaglandin Receptor and Its Application to Prostaglandin Measurements

(rat/adipocytes/antagonists/analogues/mouse ovary assay)

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Communicated by Max Tishler, December 10, 1971

ABSTRACT **A prostaglandin receptor is present in rat adipocytes, as shown by competition studies with [^{3}H]-prostaglandin E_1. The affinity of the E prostaglandins for this receptor preparation is greater than that of A and F prostaglandins, an observation consistent with their relative potencies in stimulating cyclic AMP formation in isolated mouse ovaries and various other organs. In the protocol described, binding constants of 3–8 nM were obtained with prostaglandins E_2 and E_1. A sensitivity of 1 pmole makes this method readily applicable for measurement of tissue concentrations of the E prostaglandins.**

There has been no direct documentation of the existence of a prostaglandin receptor site. However, its presence is implied by the physiological (1) and biochemical (2) effects elicited by the prostaglandins that could be blocked by antagonists in a competitive manner. Our search for a binding assay for the prostaglandins was begun because of the finding of a distinct dose–response relationship between added prostaglandins and cyclic AMP formation in isolated mouse ovaries, a response that is blocked in a competitive manner by the prostaglandin antagonist, 7-oxa-13-prostynoic acid (2, 3). Binding studies with mouse ovaries were not extensively pursued, because our initial efforts were unpromising. Rather, our attention was drawn to rat lipocytes, which are readily obtainable in homogeneous form and in addition are uniquely sensitive to the action of prostaglandins. To our knowledge, lipocytes are the only cells wherein prostaglandins depress, rather than stimulate, cyclic AMP formation. However, since cyclic AMP must be formed after the binding of prostaglandin to its receptor, it is reasonable to assume that the prostaglandin receptor in adipocytes is substantially the same as that present in other cell types. This concept appears to be valid, and is the basis of the methodology for the prostaglandin assay described herein.

Bioassays (4) have usually been used to determine nanogram quantities of prostaglandins. Since these determinations depend upon physiological responses, they suffer from the obvious shortcomings of indirect measurements. Combined gas chromatography–mass spectrometry (5) has been recently used to measure nanogram quantities of the prostaglandins, but such methods require extraction from tissues and subsequent purification before analysis. Furthermore, this method only approaches the sensitivity required for routine tissue measurements. More recently, immunochemical assays have been devised to measure the prostaglandins; prostaglandin–protein conjugates (6–9) are used to prepare the antiserum. Since less than picomolar quantities of prostaglandins are measurable by this technique, this approach appears to have met the requirement of sensitivity. The problem of crossreactance does not appear to have been completely resolved in these studies, however. Furthermore, preparation of antigens and antisera with high binding characteristics are necessary for this approach.

MATERIALS AND METHODS

[5,6-^{3}H]Prostaglandin E_1 (PGE_1) (Lot Number 509-203, 83 Ci/mmol) was purchased from New England Nuclear Corp. and used in these studies without further purification. The Male Holtzman rats (220–300 g) were maintained on Purina chow *ad libitum* and were killed by decapitation. Bacterial collagenase (CLS) was purchased from Worthington Biochemical Corp.

Lipocyte suspensions were prepared by a modification of the Rodbell method (10). 2 g of epididymal fat pads were digested with 20 mg of collagenase for 60 min at 37°C in 5 ml of Tris–saline buffer [0.01 M Tris·HCl (pH 7.5)–0.15 M NaCl]. The lipocytes were washed with two 5-ml portions of this buffer (which was used throughout these studies), and finally suspended in buffer to make a total volume of 5 ml. The lipocyte suspension was strained through cheesecloth and then homogenized with eight strokes of a motor-driven glass-to-glass, Dual (Kontes Glass Co.) homogenizer. The homogenate was centrifuged at 1000 × *g* for 5 min at 25°C. The pellet and aqueous infranatant were aspirated from beneath the lipid cake and its clear oily supernatant. The clear oil phase was withdrawn from the lipid cake, which was next suspended in 3 ml of buffer, centrifuged, and separated as described initially. This washed lipid cake was suspended in 1 ml of buffer, and 0.1-ml aliquots of this suspension, containing 80–200 μg of protein, were used in the binding assay.

Each incubation mixture included 0.1 ml of the binding preparation, 0.05 ml of buffer containing 0.1 μCi [5,6-^{3}H]-PGE_1 (0.4 ng), and prostaglandin or test substances in 10 μl of methanol or dimethylsulfoxide. Incubations were at 37°C for 60 min, unless otherwise indicated. 1 ml of buffer was then added to the incubation mixture, and this suspension was applied to a small, buffer-moistened glass-wool column. Columns for this purpose were prepared by insertion of glass wool, compressed to 1-cm length, into 14.6 cm disposable Pasteur pipettes. The columns were then washed with two successive 0.5-ml portions of buffer, and the residual buffer was expelled by air pressure. A 2.5-cm segment of the column, containing the glass-wool plug, was separated with

a glass-cutting file. The plug containing the lipid material, including the bound prostaglandins, was expelled with a glass rod and deposited, along with the glass segment, into an ethanol–toluene 30:70 phosphor for determination of the radioactivity. The amount of radioactivity associated with incubation mixtures containing heat-denatured binding preparation (5 min at 100°C) was subtracted from all observations (nonspecific binding). The effect of the prostaglandin upon cyclic AMP biosynthesis in intact mouse ovaries was determined as described (2).

RESULTS

Binding was assayed at pH 7.5, since this is essentially physiological pH and is a pH at which the prostaglandins and receptor are stable. A typical standard curve with a saturating concentration of [^{3}H]PGE_1 is presented in Fig. 1. As with the cyclic AMP receptor (11), the binding of [^{3}H]PGE_1 to its receptor is essentially nonreversible. 2 ng of unlabeled PGE_1, which displaced 90% of the radioactivity when added simultaneously with the [^{3}H]PGE_1, displaced only 29% of the bound [^{3}H]PGE_1 when added 1 hr after the labeled prostaglandin. Thus, the simultaneous addition of a mixture of labeled PGE_1 and material to be measured is an obligatory requirement of this assay. The PGE_1 is unaltered during the binding process, since it can be extracted from the binding site in 90% yield with ethyl acetate, and can be demonstrated to be intact by thin-layer chromatography. The time course of the reaction, shown in Fig. 2, indicates that nearly maximum binding is obtained within 15 min; however, assays were routinely incubated for 1 hr to ensure equilibration. Intact lipocytes made as described herein may be used as well as homogenates, and give essentially identical affinity constants. Contamination of the binding preparation by intact lipocytes cannot be rigorously excluded, but it is unlikely that a significant amount of cells would survive the homogenization procedure. The nature of the prostaglandin receptor itself was not explored other than to show that it is labile to heat (100°C for 5 min), trypsin digestion, incubation at pH 3.5, and 10 μM *p*-chloromercuribenzoate. Binding of [^{3}H]PGE_1 to the receptor was not altered by inclusion of 0.01 M $CaCl_2$, KCl, $MgSO_4$, NaF, or NaEDTA in the incubation medium. In addition, 0.5 μM *d,l*-norepinephrine and 3 mM theophylline, concentrations that induce lipolysis in intact lipocytes, did not alter the binding of PGE_1 to the receptor preparation (data not shown).

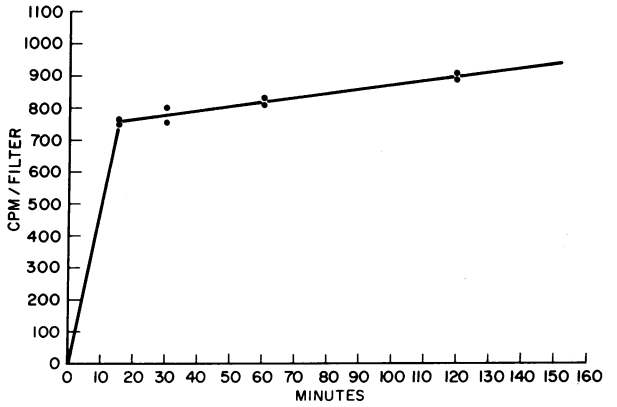


FIG. 2. Rate of association of [^{3}H]PGE_1 to binding preparation. [^{3}H]PGE_1 (0.4 ng, 0.1 μCi) was incubated with 106 μg of binding preparation protein for the indicated time at 37°C. The "bound-[^{3}H]PGE_1" was separated. The radioactivity, 100 cpm/filter of a heat-denatured preparation was subtracted from all observations.

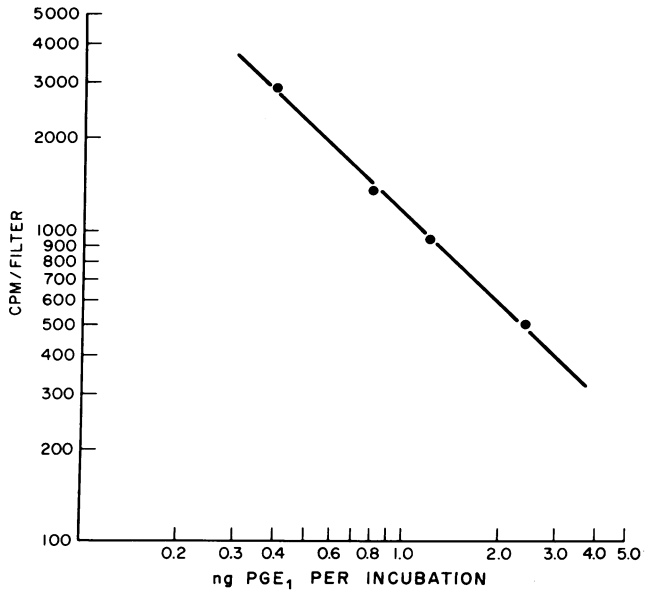


FIG. 1. Dose–response relationship for PGE_1. Each incubation contained 0.4 ng of [^{3}H]PGE_1 (0.1 μCi), and 190 μg of binding preparation protein, with additions of unlabeled PGE_1 0.4, 0.8, and 2 ng, respectively. All tubes were incubated 60 min at 37°C. [^{3}H]PGE_1 associated with the receptor preparation was separated from free [^{3}H]PGE_1. The radioactivity, 260 cpm/filter of a heat-denatured preparation was subtracted from all observations.

As indicated in Fig. 3, several representative steroids and fatty acids, even at concentrations 12,500-times greater than the added [^{3}H]PGE_1 did not prevent the binding of prostaglandin to the receptor site. At these same high concentrations, however, arachidonic acid did show some affinity for the receptor. The possibility that this prostaglandin precursor may be converted biosynthetically to PGE_2 during the incu-

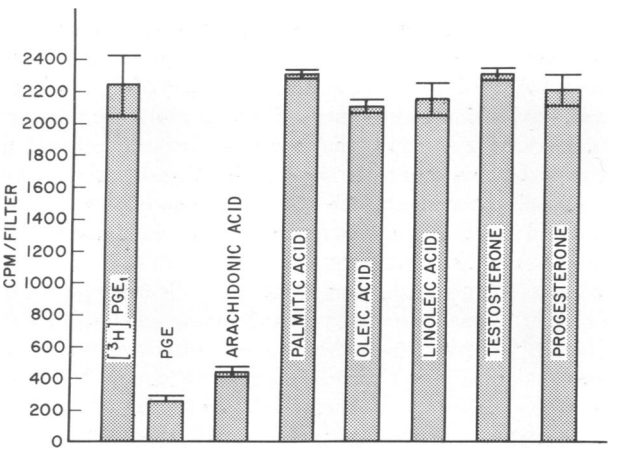


FIG. 3. The effect of various lipids on PGE_1 binding. Each incubation contained 0.4 ng of [^{3}H]PGE_1 and 156 μg of binding preparation protein, plus additions of 2 ng PGE_1 and 5,000 ng of various fatty acids and steroids. All tubes were incubated 60 min at 37°C. [^{3}H]PGE_1 associated with the receptor preparation was isolated, and a blank value of 160 cpm/filter was subtracted.

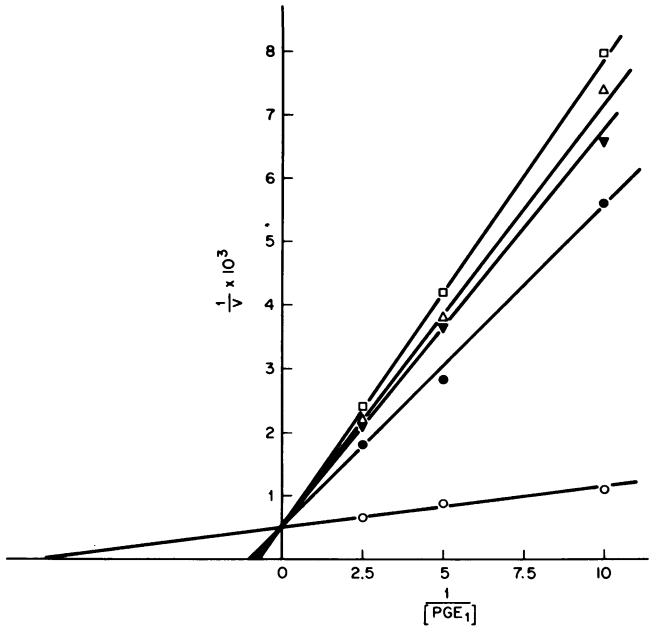


FIG. 4. Competitive interaction of various prostaglandins. [^{3}H]PGE_1, 0.1, 0.2, and 0.4 ng, was incubated at 37°C for 60 min with 123 μg of binding preparation in the presence or absence of various prostaglandins (PGE_2, 1 ng; $PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGA_1, 100 ng). Appropriate blank values were subtracted from all observations. V = cpm [^{3}H]PGE_1 per filter per 60 min. O——O, PGE_1; △——△, PGE_2; □——□, $PGF_{2\alpha}$; ●——●, $PGF_{1\alpha}$; ▼——▼, PGA_1.

bation cannot be excluded. However, inclusion in the incubation of the prostaglandin biosynthesis inhibitor, fluoroindocin (12), which by itself does not compete with prostaglandin for the receptor site, did not alter the displacement by arachidonic acid, suggesting that this fatty acid may have a real, if weak, affinity for the receptor (data not shown).

In Fig. 4, the concentration of [^{3}H]PGE_1 was varied in the presence and absence of various prostaglandins. By Lineweaver–Burk plots, binding constants for PGE_1 and PGE_2 of 3 nM and $PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGA_1 of 300 nM were obtained. The amount of prostaglandin bound to the receptor preparation was of the order of 20 pg per tube. The relative potencies of various prostaglandins with respect to PGE_1 are presented in Table 1. It is apparent that there is substantial correlation between the ability of these substances to bind to the lipocyte receptor preparation and the ability to stimulate cyclic AMP formation in intact mouse ovaries. In Fig. 5, the binding properties of several known prostaglandin antagonists are shown, including polyphloretin phosphate (13), 7-oxa-13-prostynoic acid (2,3), 7-oxa-15-hydroxy-13-prostynoic acid (2, 3), and SC19220 [1-acetyl-2-(8-chloro-10,11-dihydrodibenz[*b*,*f*] [1, 4] oxazepine-10-carbonyl)hydrazine] (14). Of the four, only the structures related to prostaglandin bind to the receptor, but the concentrations required are markedly higher than those of the prostaglandins themselves for equivalent displacement of [^{3}H]PGE_1 from the binding site.

DISCUSSION

The advantages of the procedure described for the measurement of prostaglandins of the E-type are specificity, sensitivity, and ease of manipulation. Furthermore, the fact that the major initial degradation product derived from PGE, the 15-keto derivative, has no significant affinity for the prostaglandin receptor precludes its interference with this assay. To our knowledge, the absence of crossreactance has not been demonstrated for biologically inactive prostaglandin metabolites in the available radioimmunochemical assays. The weak affinity of the F-type prostaglandins for the lipocyte receptor remains unexplained, but does correlate with the relatively small response elicited by these prostaglandins in stimulating cyclic AMP formation in the mouse ovary and other tissues (12). It is possible that the response to F-type prostaglandins in both of these assays is due to contamination by trace amounts of the E-prostaglandins. In any event, the extreme affinity of the E-type prostaglandins for the lipocyte receptor, compared to the A and F-series, effectively excludes measurement of the latter by this binding technique in the presence of even trace amounts of the E-prostaglandins. This assay, in evaluating a tissue mixture of prostaglandins, should closely reflect their combined ability to stimulate cyclic AMP formation.

Prostaglandin $F_{2\alpha}$ exerts a potent physiological response in the vein, lung, and uterus (15). It is difficult, however, to account for its biological activity upon the basis of its relatively weak affinity for the lipocyte receptor, as well as its minimal effectiveness in increasing cyclic AMP in many tissues (including the uterus). Thus, the existence of a receptor unique to the PGFs in certain tissues deserves consideration. Alternatively, the F-type prostaglandins could function as antagonists to the action of endogenous PGEs under certain conditions, and exert their physiological response in this manner. Although we have been unable to demonstrate an antagonistic effect of $PGF_{2\alpha}$ upon PGE_2-induced formation of cyclic AMP *in vitro*, in either the mouse ovary or rabbit uterus (data not shown), such an antagonism between PGE_1 and $PGF_{2\alpha}$ has been demonstrated on the contractile response of the human bronchial muscle (16) and cardiac output in dogs (17). It is of interest to note that increased concentrations of cyclic AMP appear to be associated with relaxation of the rat uterus (19).

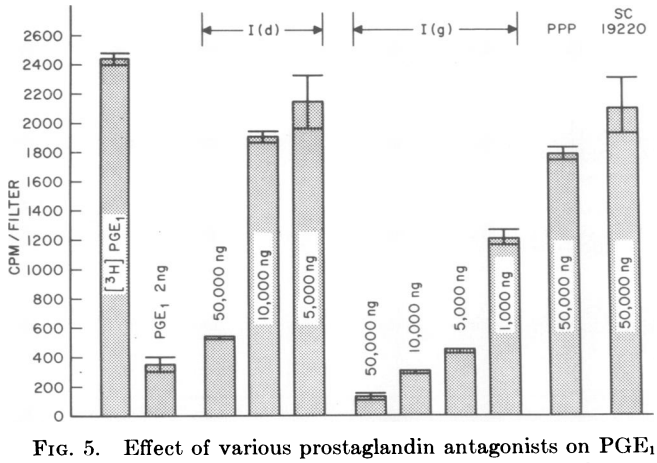
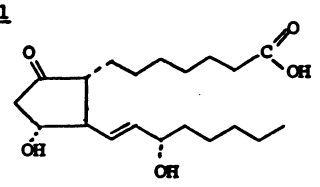
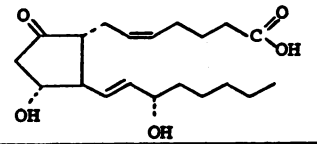
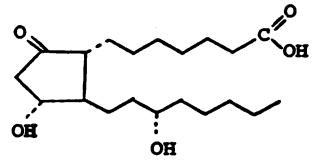
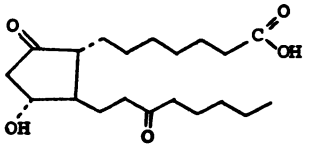
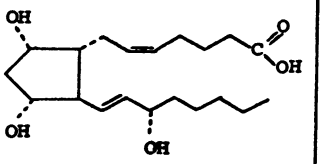
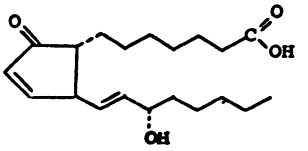


FIG. 5. Effect of various prostaglandin antagonists on PGE_1 binding. Each incubation contained 0.4 ng [^{3}H]PGE_1 and 77 μg of binding preparation protein, plus additions of PGE_1 and prostaglandin antagonists as indicated. Experimental conditions were identical to those described in Fig. 3. The blank, 119 cpm/filter, was subtracted. *I(d)*, 7-oxa-13-prostynoic acid; *I(g)*, 7-oxa-15-hydroxy-13-prostynoic acid; *PPP*, polyphloretin phosphate; *SC 19220* (see text).

TABLE 1. *Comparison of the ability of various prostaglandins to bind to the receptor preparation and to stimulate cyclic-AMP*

Compound	Compound Conc. ng	ngm PGE_1 Equivalents	Relative Activities: ng PGE_1 Equivalents / ng Compound	Mouse Ovaries Cyclic-AMP Accumulation: Fold Stimulation over control (5 μg /ml)	K_m Molar
PGE_1 	1 2	1 2	1 1	20X	2×10^{-6}
PGE_2 	1 2	0.6 1.3	0.6 0.7	·17X	4×10^{-7}
13,14-Dihydro PGE_1 	1 2	0.2 0.4	0.2 0.2	17X	—
9,15-Diketo-11-hydroxy prostanoic acid 	500 100	2 0.5	0.004 0.005	4.2X	—
$PGF_{2\alpha}$ 	100 50	0.9 0.4	0.009 0.008	2.9X	2×10^{-4}
PGA_1 	100 50	1.2 0.7	0.012 0.014	4.7X	—

Appropriate concentrations of the various prostaglandins were incubated with 0.4 ng of [^{3}H]PGE_1 and 125 μg of binding preparation protein for 60 min at 37°C. The amount of [^{3}H]PGE_1 associated with the receptor preparation was determined as described in *Methods*. The radioactivity of the heat-denatured controls was subtracted from all observations. The mass of PGE_1 equivalent to a given concentration of compound was determined, and expressed in the above table as "PGE_1 equivalents." The effects of the prostaglandins, 5 μg/ml, on cyclic AMP accumulation in intact ovaries and the K_m values for this activation were determined.

The nature of the prostaglandin receptor was not extensively explored in these studies. Nevertheless, the affinity of the crude binding fraction for the lipid layer of fat-cell homogenates, and its inactivation by both *p*-chloromercuribenzoate and trypsin, does permit the suggestion that the prostaglandin receptor site is a sulfhydryl-containing lipoprotein. In addition, the close correlations between the affinity of the various prostaglandins for the lipocytic receptor and their ability to stimulate cyclic AMP formation in various tissues (12) is compatible with the concept that the prostaglandin receptor is similar in all these target organs. Since PGE_2, PGE_1, and 13,14-dihydro PGE_1 bind to about the same degree, it is apparent that the amount of unsaturation in the side chain is not a significant factor in the initial binding reaction. Thus, the finding that the potency of the E-type prostaglandins in stimulating cyclic AMP formation in isolated mouse ovaries is diminished with decreased unsaturation (i.e., $PGE_2 > PGE_1 >$ 13,14-dihydro PGE_1) suggests that this structural feature is allosterically involved in the activation of adenylate cyclase. We may speculate further that tissue specificity may be largely concerned with the degree of unsaturation of the side chain, since it is well established that PGE_1 is more potent than PGE_2 in preventing platelet aggregation (20), whereas the reverse is true in the case of ovarian steroidogenesis (12). Nevertheless, it is not possible to discount the importance of the integrity of the prostaglandin side-chain for the binding phenomenon, since the conversion of the 15-hydroxy group to a carbonyl function almost completely obliterates this response. This observation is in accord with the marked decrease in prostaglandin activity associated with the metabolic conversion of the 15-hydroxy group to a carbonyl function.

Although the ultimate physiological or biochemical response of a prostaglandin may be blocked in a competitive manner at any of the sequential steps subsequent to the "binding phenomenon" (18), a true antagonist (in the pharmacological sense) must act by competing for the prostaglandin receptor site. Accordingly, a number of "prostaglandin antagonists" were examined to see if they fulfill this requirement. The finding that 7-oxa-13-prostynoic acid does, in fact, compete for the receptor adds substance to the assertion (2, 3) that this compound is a prostaglandin antagonist. The high concentration of this compound required to elicit this effect is some cause for concern in judging the significance of the binding phenomenon. Nevertheless, the close relative of this antagonist, 7-oxa-15-hydroxy-13-prostynoic acid, has only slightly superior binding characteristics and is a prostaglandin mimic in the mouse-ovary cyclic AMP assay. It is difficult to visualize that such a compound could elicit a stimulatory effect other than by binding to the prostaglandin receptor. The observation that polyphloretin phosphate, even at high concentrations does not measurably diminish the binding of [^{3}H]-PGE_1 to the receptor is compatible with the recent report that this substance acts after binding has taken place (18). SC19220 has no affinity for the prostaglandin receptor site, so the nature of its antiprostaglandin activity remains unexplained.

We thank R. Hoffsommer, D. Taub, and N. Wendler of the Merck Sharp and Dohme Research Laboratories for preparing the prostaglandin derivatives and B. Lopez-Ramos for the protein determinations. We are grateful to Dr. J. Fried of the University of Chicago for the supplies of Id and Ig (see Fig. 5) and Dr. K. Eakins, Columbia University, for the sample of polyphoretin phosphate.

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