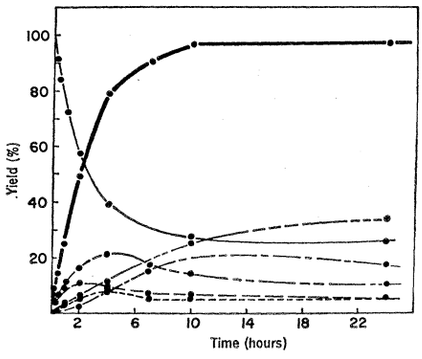


Fig. 6. Phosphorylation of uridine with a mixture of inorganic phosphate, ammonium chloride, ammonium bicarbonate, and urea at 100°C. U, ————; Up!, — - - - —; pU, — - - —; Up, — — — —; pUp!, — - —; pUp - - - - -; total incorporation of inorganic phosphate, ————.

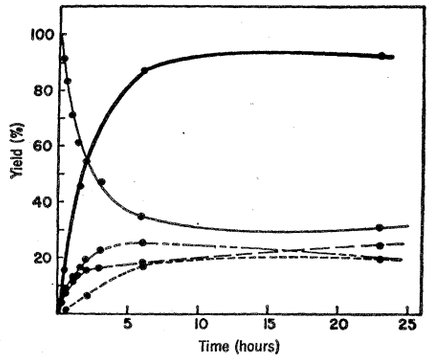


Fig. 7. Phosphorylation of thymidine with a mixture of inorganic phosphate, ammonium chloride, ammonium bicarbonate, and urea at 100°C. T, ————; pT, — - - —; Tp, — — — —; pTp - - - - -; total incorporation of inorganic phosphate, ————.

ammonium chloride limits the extent of carbamylation.

It is doubtful that temperatures as high as 100°C occurred at many points on the primitive earth. We therefore carried out a few experiments at lower temperatures. The rate of reaction was then much decreased but still quite significant. For example, after 4 days at 66°C we were able to detect 9.6 percent combined uridine phosphates and 4 percent Up! from the ammonium chloride–urea mixture. Surface temperatures above 70°C are quite realistic since they are reached today in many desert areas and also in loose-packed beach sand.

Finally, we carried out experiments in which phosphate is present as hydroxylapatite, since this is the form of phosphate found most often in nature. After 24 hours at 100°C 16 percent combined uridine phosphates and 4 percent Up! were obtained (Table 3).

The rates of solid-state reactions are extremely sensitive to the physical state of the reactants. Hence the quantitative details described here are probably not important. Reactions on inert materials with larger surface areas would probably be more efficient. Reactions on the surface of a glass tube are slower but go to completion after a few hourly cycles of moistening and reheating to 100°C. The incomplete conversion of guanosine to phosphates may well be due to its low solubility. The main point we wish to make is that high yields can certainly be obtained under mild conditions.

We have preliminary evidence that condensed inorganic phosphates are formed in good yield from inorganic phosphate, ammonium chloride, and urea under the reaction conditions that we employed. However, we have no evidence that condensed inorganic phosphates play any part in the reaction. Organic phosphates such as carbamyl phosphate or phosphoramidates may be the true active intermediates.

We believe that the reactions described here constitute a completely plausible model of prebiotic phosphorylation. They proceed at temperatures of 100°C in a few hours or 66°C in a few days. Yields of a few tens of percent can be obtained from an equimolar mixture of soluble phosphate and a nucleoside. Hydroxylapatite can be substituted for sodium phosphate with only a moderate (fivefold) decrease in yield. The synthesis we describe could easily have occurred either on beach sand or in desert areas where temperatures in excess of 70°C are encountered.

The synthesis of urea under prebiotic conditions has been reported many times (*14*). Irradiation of ammonium cyanide solutions gives yields of up to 30 percent (*15*). Thus the requirement for urea is no obstacle to this proposed prebiotic reaction sequence.

R. LOHRMANN
L. E. ORGEL
Salk Institute for Biological Studies, San Diego, California 92112

References and Notes

1. See D. H. Kenyon and G. Steinman, *Biochemical Predestination* (McGraw-Hill, New York, 1969), chap. 5.
2. R. Lohrmann and L. E. Orgel, *Science* **161**, 64 (1968).
3. A. Beck, R. Lohrmann, L. E. Orgel, *ibid.* **157**, 952 (1967).
4. M. Halmann, R. A. Sanchez, L. E. Orgel, *J. Org. Chem.* **34**, 3702 (1969).
5. V. W. Feldmann, *Chem. Ber.* **100**, 3850 (1967).
6. A. W. Schwartz, *Chem. Commun.* **1969**, 1393 (1969).
7. R. Saffhill, *J. Org. Chem.* **35**, 2881 (1970).
8. K. O. Knollmueller, U.S. Patent 3.333921; *Chem. Abstr.* **67**, 74990c (1967); F. O. Lundstrom and C. W. Whittaker, *Ind. Eng. Chem.* **29**, 61 (1937).
9. G. C. Daul and J. D. Reid, U.S. Patent 2.609360; *Chem. Abstr.* **47**, 920h (1953).
10. Ribonuclease T_2 was supplied by Dr. G. W. Ruchizky.
11. M. Smith, J. G. Moffatt, H. G. Khorana, *J. Amer. Chem. Soc.* **80**, 6204 (1958).
12. Abbreviations: U, uridine; T, thymidine; A, adenosine; G, guanosine; C, cytidine; Xp!, ribonucleoside cyclic 2′,3′-phosphate; pX, nucleoside 5′-phosphate; Xp, nucleoside 2′(3′)-phosphate; pXp!, 5′-phosphonucleoside cyclic 2′.3′-phosphate; pXp, nucleoside 5′,2′(3′)-diphosphate; x, base of the corresponding nucleoside X; and U*, carbamylated uridine.
13. An anhydrous solution containing thymidine (0.2 mmole), pyridinium cyanoethylphosphate (0.8 mmole), and 2.4,6-triisopropylbenzene sulfonylchloride (1.6 mmole) in pyridine (4 ml) was kept for 3 hours at room temperature. Subsequently, the mixture was diluted with water (4 ml), and left standing for another 15 minutes. After addition of 10*N* NH_4OH (30 ml), the mixture was maintained under pressure at 80°C for 18 hours. It was then evaporated under vacuum and chromatographed in system VI. Elution of the strong ultraviolet-absorbing band gave the ammonium salt of pTp (74 percent yield).
14. M. Labadie, R. Jensen, E. Neuzil, *Bull. Soc. Chim. Biol.* **49**, 673 (1967); J. E. Varner and R. C. Burrell, *Euclides* **15**, 1 (1955); *Chem. Abstr.* **49**, 11735h (1955); C. U. Lowe, M. W. Rees, R. Markham, *Nature* **199**, 219 (1963); C. Ponnamperuma, *Proceedings of the Third International Radiation Research Congress*, G. Silini, Ed. (North-Holland, Amsterdam, 1967), p. 700.
15. R. Lohrmann and L. E. Orgel, in preparation.
16. We thank S. Khoury for technical assistance. Work supported in part by NASA grant NGR 05-067-001 and NIH grant 13435.

10 November 1970

Radioimmunoassay for Prostaglandins

Abstract. *Antibodies to prostaglandin were obtained by immunization of rabbits with PGA_1, PGA_2, and PGE_1 protein conjugates of prostaglandins. The antibodies demonstrated specificity toward both the cyclopentane ring and the aliphatic side chains. With the use of these antibodies a highly sensitive radioimmunoassay capable of measuring less than picomolar amounts of PGA_1, PGA_2, and PGE_1 has been developed.*

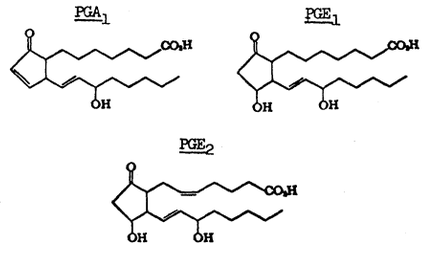
The prostaglandins are 20-carbon aliphatic carboxylic acids with a cyclopentane ring; their molecular weights range from 332 to 356. They are widely distributed in biological systems, and physiologic studies have indicated diverse effects on smooth muscle tone (*1*), blood pressure (*2*), lipolysis (*3*), platelet aggregation (*4*), and central nervous system function (*5*)—which

may be mediated through the adenyl cyclase system (*3*). Confirmation of the importance of these fatty acids has been hampered by a lack of a suitable method for their measurement. Bioassays (*6*) have been employed to determine nanogram concentrations of prostaglandins, but these depend on physiologic responses rather than on measuring the compounds directly. The chemical methods available are useful in the microgram range (*7*). We now describe the production of antibodies to prostaglandins and the development of a radioimmunoassay capable of measuring low picogram amounts of major prostaglandin classes.

The antibodies described were induced by immunization of rabbits with prostaglandin-protein conjugates. Four milligrams of prostaglandin (PG) A_1, A_2, or E_1 (*8*) dissolved in 4.0 ml of 10 percent ethanol–0.02 percent aqueous sodium carbonate were reacted with 8 mg of human serum albumin in the presence of 4 mg of 1-ethyl-3-(3-diethylaminopropyl)carbodiimide hydrochloride at *p*H 5.5 (*9*). The reaction was carried out for 24 hours at 20°C and then the product was exhaustively dialyzed against a buffer of 0.15*M* NaCl and 0.01*M* phosphate, *p*H 7.45 (PBS). Conjugation of prostaglandin to the protein carriers was confirmed by observing an increase with time in absorption at 278 nm at alkaline *p*H (*7*), as compared with control protein solutions. From the known molar extinction coefficient ($E_m = 27{,}200$) (*7*) the degrees of substitution of PGE_1, PGA_1 and PGA_2 were estimated as 3.3, 2.7, and 1.5 moles of prostaglandin per mole of protein (*10*), respectively. PGE_1 was also conjugated to keyhole limpet hemocyanin (KLH) at 10°C with the use of ethyl chloroformate (*11*). After 2 mg of PGE_1 was mixed for 15 minutes with equimolar quantities (with respect to PGE) of ethyl chloroformate and triethylamine (0.9 and 0.6 μl, respectively) in 30 μl of dioxane, 5.5 mg of KLH was added in 0.45 ml of 0.1*N* $NaHCO_3$, and the reaction mixture was stirred for 1 hour more before dialysis with PBS.

Dialyzed conjugates were emulsified with equal volumes of complete Freund's adjuvant (*12*). At the initial immunization, rabbits received 1 mg of immunogen subcutaneously (0.25 mg per foot pad). At intervals of 2 to 4 months animals were given booster injections of 50 to 150 μg in complete adjuvant. Ten days after the booster injection, the animals were bled and their serums were examined for the presence of antibody activity to prostaglandins.

Table 1. Globulin fractions of the antiserums listed in the left hand column were incubated with [^{3}H]PGE_1 (6000 count/min) (*14*). The prostaglandins listed were added in 50-ng amounts to evaluate comparative inhibition of the binding of tritiated label.



Antiserum to	Inhibition of bound [^{3}H]PGE_1 in the presence of 50 ng of		
	PGA_1	PGE_1	PGE_2
PGA_1-HSA	54	35	20
PGA_2-HSA	46	0	8
PGE_1-KLH	42	40	32

With this immunization scheme, all of the rabbits produced antibody to prostaglandins. Compared with normal rabbit serum, globulin fractions (40 percent ammonium sulfate fractionation and diethylaminoethyl-cellulose chromatography) (*13*) from serums containing *early* antibody bound up to 20 percent of a [^{3}H]PGE_1 of relatively low specific activity (*14*). The addition of 20 to 20,000 ng of unlabeled PG significantly inhibited the binding of label (see below) in all cases. Specificity was directed both against the cyclopentane ring (Table 1) (which distinguishes PGA, PGE, and PGF from one another) and the side chains (as influenced by the presence or absence of double bonds at C 5-6, which distinguishes PGA_2 and PGE_2 from PGE_1 and PGA_1). In experiments with early antiserums (Table 1) PGA_1, PGE_1, and PGE_2 inhibited binding of [^{3}H]PGE_1 to different degrees, depending on the immunogen. The homologous compounds were the most effective inhibitors; the apparent inability of antibody to PGE_1 to distinguish between PGA_1 and PGE_1 may have been due to conversion of protein bound PGE to PGA in vivo or in vitro.

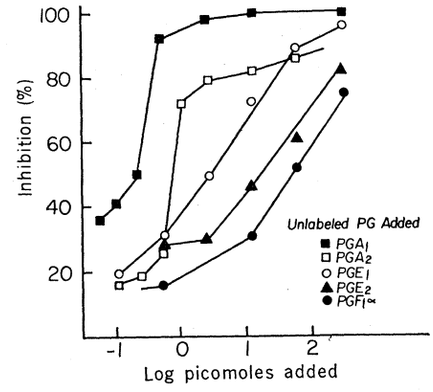


Fig. 1. Antiserum to PGA_1 was incubated with [^{3}H]PGE_1 (*15*) in the absence and presence of various concentrations of prostaglandins. Antibody-bound [^{3}H]PGE_1 was separated from unbound by ammonium sulfate precipitation. Percentage inhibition was calculated from the difference in binding in the presence and absence of inhibitor.

Serums obtained 12 to 16 months after the initiation of immunization have considerably higher titers. With a [^{3}H]PGE_1 of higher specific activity (*15*) 50 μl of a 1 : 250 dilution of serum binds 90 percent of the tritiated marker; 50 μl of a 1 : 10,000 dilution of an antiserum to PGA_1 binds 50 percent of the label.

The better binding has resulted in the development of a sensitive radioimmunoassay system for prostaglandins. In the current assay system antibody (1 : 5000 dilution of serum), normal rabbit γ-globulin (*16*), unlabeled prostaglandin (0.05 to 333 pmole) and high specific activity [^{3}H]PGE_1 are incubated together in polypropylene test tubes (final volume, 0.3 ml) at 4°C for 72 hours. Antibody-bound [^{3}H]PGE_1 is separated from unbound label by ammonium sulfate precipitation to a final concentration of 50 percent of saturation. The washed precipitate is dissolved in Nuclear-Chicago solubilizer (NCS) and counted in toluene scintillation solution. The current sensitivity of the immunoassay system is less than 0.1 pmole (30 pg) for PGA_1 and less than 0.15 pmole for PGE_1. Figure 1 illustrates a comparative inhibition study with an antiserum specific for PGA_1. In the absence of inhibitor, 40 percent of the tritiated label was specifically bound by antibody. Inhibition was carried out by addition of equimolar amounts of the various prostaglandins indicated. Percentage inhibition was calculated from the difference in binding in the presence and absence of unlabeled prostaglandin. In the experiment demonstrated, PGA_1, the homologous ligand, inhibited significantly when less than 0.1 pmole was added. The comparative degrees of inhibition again demonstrated that the

antibody used had specificity directed toward both the cyclopentane ring ($PGA_1 > PGE_1 > PGF_{1\alpha}$) and the side chains ($PGA_1 > PGA_2$ and $PGE_1 > PGE_2$).

This prostaglandin immunoassay system has not yet been fully characterized. However, it is already clear that by varying the antibody PGA_1, PGA_2, and PGE_1 can be measured in subpicomolar amounts. The present sensitivity of the immunoassay system and the differing degrees of prostaglandin cross reactivity justify the prediction that the radioimmunoassay system will be useful in evaluating the physiologic role of various members of the prostaglandin family.

BERNARD M. JAFFE, JAY W. SMITH
WILLIAM T. NEWTON
CHARLES W. PARKER
Departments of Surgery and *Medicine, Washington University School of Medicine, St. Louis, Missouri 63110*

References and Notes

1. E. W. Horton and I. H. M. Main, *Brit. J. Pharmacol. Chemother.* **24**, 470 (1965).
2. J. B. Lee, B. G. Covino, B. H. Takman, E. R. Smith, *Circ. Res.* **17**, 57 (1965).
3. D. Steinberg, M. Vaughan, P. J. Nestel, O. Strand, S. Bergström, *J. Clin. Invest.* **43**, 1533 (1964); R. W. Butcher, J. E. Pike, E. W. Sutherland, in *Proceedings of the 2nd Nobel Symposium*, S. Bergström and B. Samuelsson, Eds. (Almqvist & Wiksell, Stockholm, 1967), p. 133.
4. J. Kloeze, in *Proceedings of the 2nd Nobel Symposium*, S. Bergström and B. Samuelsson, Eds. (Almqvist & Wiksell, Stockholm, 1967), p. 241.
5. G. L. Avanzino, P. B. Bradley, J. H. Wolstencroft, *Brit. J. Pharmacol. Chemother.* **27**, 157 (1966).
6. S. Bergström, L. A. Carlson, J. R. Weeks, *Pharmacol. Rev.* **20**, 1 (1968).
7. J. E. Shaw and P. W. Ramwell, *Methods Biochem. Anal.* **17**, 325 (1969).
8. We thank Drs. John E. Pike and Wayne Magee of the Upjohn Company, Kalamazoo, Michigan, for gifts of PGA_1, PGA_2, PGE_1, PGE_2, and [^{3}H]PGA_1.
9. J. C. Sheehan and J. J. Hlavla, *J. Org. Chem.* **21**, 439 (1956); B. M. Jaffe, W. T. Newton, J. E. McGuigan, *Immunochemistry* **7**, 715 (1970).
10. The molecular weight of human serum albumin was taken as 70,000.
11. J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids* (Wiley, New York, 1961), vol. 2, p. 978; G. C. Oliver, B. M. Parker, D. L. Brasfield, C. W. Parker, *J. Clin. Invest.* **47**, 1035 (1968).
12. C. W. Parker, S. M. Godt, M. C. Johnson, *Biochemistry* **5**, 2314 (1966).
13. ———, *ibid.* **6**, 3417 (1967).
14. [^{3}H]PGE_1 was prepared in our laboratory by catalytic reduction of PGE_2 to PGE_1 with the use of tritium gas followed by preparative thin-layer chromatography. Specific activity < 1 c/mmole; B. Samuelsson, *J. Biol. Chem.* **239**, 4091 (1964).
15. Obtained from New England Nuclear Corporation, Boston, Massachusetts, at specific activities of 28 and 87.5 c/mmole.
16. Normal rabbit gamma globulin was added as carrier to ensure maximal precipitation of antibody.
17. Supported by NIGMS training grant GM 371 and grant AI 00219.

18 November 1970

Adenosine 3′,5′-Monophosphate Phosphodiesterase in the Growth Medium of Physarum polycephalum

Abstract. *The acellular slime mold* Physarum polycephalum *releases a soluble adenosine 3′,5′-monophosphate phosphodiesterase into the growth medium. Although this enzyme resembles the particulate diesterase of the same organism in kinetic properties and in inhibition by methyl purines, its greater stability, its insensitivity to stimulation by imidazole and to inhibition by adenosine triphosphate, and its selective release into the medium indicate a specific function, perhaps protection against exogenous cyclic nucleotide, for the soluble enzyme.*

Although adenosine 3′,5′-monophosphate (3′,5′-AMP) has been studied extensively in animal tissues as the probable mediator of the action of many hormones (*1*), recent work has also implicated 3′,5′-AMP in organisms not known to respond to hormones. Examples include the control of enzyme synthesis in bacteria (*2*) and the stimulation of aggregation (*3, 4*) and possibly differentiation (*5*) of *Dictyostelium discoideum* amoebae. Thus, in bacteria and in slime molds, 3′,5′-AMP appears to have highly specialized functions related to normal growth and development. The slime molds in particular may normally be expected to exist in environments providing exogenous sources of 3′,5′-AMP (for example, from dead bacteria). Because an exogenous supply of this nucleotide might interfere with normal regulatory mechanisms, such organisms may have evolved protective mechanisms. Thus *Dictyostelium* amoebae release into the growth medium a phosphodiesterase that hydrolyzes 3′,5′-AMP to 5′-AMP (*6*).

The present results show that *Physarum polycephalum* plasmodia contain a largely particulate 3′,5′-AMP phosphodiesterase and also release a potent phosphodiesterase into the growth medium; the properties of the two enzymes are significantly different.

Physarum polycephalum was grown in submerged culture (*7*); 30 ml of growth medium was inoculated with about 1 ml of a 3-day culture. When required, surface cultures were established on filter paper in contact with liquid medium (*8*). To obtain material for enzyme extraction, the mold was allowed to settle in the culture flask, collected with a Pasteur pipette, and washed by again settling through fresh medium. After removal of excess medium on filter paper, the mold was homogenized for 30 seconds in a Potter-Elvehjem homogenizer in 50 m*M* tris(hydroxymethyl)aminomethane (tris) (*p*H 7.4) and centrifuged at 20,000*g* for 10 minutes. The residue was washed once in 50 m*M* tris (*p*H 7.4) and finally suspended in the same buffer. To test for extracellular phosphodiesterase the growth medium was separated from the mold by decanting after 3 days of growth in shake culture and centrifuged at 20,000*g* for 15 minutes. The phosphodiesterase in the growth medium could be concentrated by precipitation between 40 and 60 percent saturation with ammonium sulfate followed by dialysis against 500 volumes of 10 m*M* tris (*p*H 7.4) for 16 hours; unless otherwise stated this concentrated enzyme was used in all studies with the growth medium enzyme.

Enzyme activity was routinely assayed by separation of the reaction products by paper chromatography. The reaction mixture contained, in a final volume of 0.03 ml, 0.05*M* tris buffer (*p*H 7.4), 0.01*M* $MgCl_2$, 0.35 μc of tritiated 3′,5′-AMP (specific activity, 1.4 c/mmole), approximately 10 μg of snake venom 5′-nucleotidase, 2.9 m*M* 3′,5′-AMP unless otherwise stated, and enzyme. Incubations were carried out at 30°C, and reactions were stopped with 0.01 ml of concentrated HCl. Samples (0.05 ml) were chromatographed on Whatman 3-MM paper in a solution of 1*M* ammonium acetate and ethanol (15 : 35, by volume); radioactivity associated with adenosine was measured by liquid scintillation counting. Control assays indicated that the 5′-nucleotidase preparation did not contain significant 3′,5′-AMP phosphodiesterase activity. The activity of 5′-nucleotidase was measured in similar assays without 3′,5′-AMP and 5′-nucleotidase and including 0.4 m*M* [8-^{14}C]AMP (specific activity, 1.2 μc/μmole). Protein was measured by the method of Lowry *et al.* (*9*).

Preliminary experiments indicated that 3′,5′-AMP phosphodiesterase activity was mainly located in a particulate fraction of *Physarum* and was also