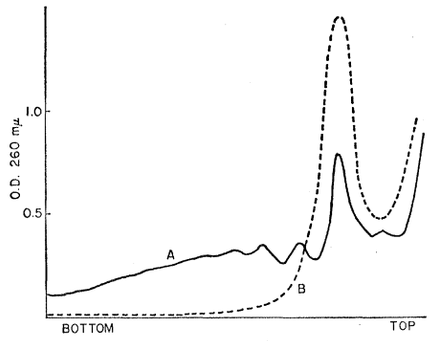

Fig. 1. Optical densities at 260 mμ. A preparation of polysomes from *E. coli* was placed on a cold, 26-ml, linear, 15- to 30-percent sucrose gradient (0.01*M* in tris hydrochloride, *p*H 7.6, and 0.01*M* in magnesium acetate) and was spun for 150 minutes at 25,000 rev/min in a Spinco-SW25 rotor at 5°C. The gradient was analyzed (*A*) with a Gilford continuously recording spectrophotometer. To test the sensitivity of the polysomes to ribonuclease, this enzyme (Worthington Biochemical Corp., Freehold, N. J.) was added at 5 μg/ml to one portion of the *A* preparation at the time of addition of deoxycholate, and the preparation was similarly analyzed (*B*).

gation (Fig. 1, curve *A*). The polysomes appeared as a broad peak, preceding the 70*S* peak and amounting regularly to 50 to 75 percent of the total ribosomes in the extract. The percentage of polysomes in several samples of a given batch of cells was reproducible to within 5 percent.

The polysome peak does not represent either fragments of DNA or aggregates of ribosomes with the basic protein lysozyme, since treatment of the extract with ribonuclease (5 μg/ml) at 0°C for several minutes completely eliminated the peak and correspondingly increased the 70*S* peak (Fig. 1, curve B). A similar shift was observed when the supply of messenger RNA in the cells was depleted by incubating *Micrococcus lysodeikticus* with actinomycin D (10 μg/ml) for 20 minutes. Results were much the same when *E. coli* was incubated with proflavin (*4*).

The yield of ribosomes was estimated by dividing a lysing suspension into three equal portions, treating one portion with ribonuclease (5 μg/ml) at 0°C for a few minutes to digest the messenger RNA, and sonicating the second portion for 1 minute to release all ribosomes from all the cells; the third portion was left untreated. The cell debris was removed by centrifugation as described. The ribonuclease treatment increased the yield of RNA in the supernatant [measured as OD_{260} (optical density at 260 mμ)] by 25 percent; the difference presumably represents ribosomes trapped in the debris as polysomes. Sonication increased the OD_{260} of the supernatant by only 15 percent (above the value obtained with ribonuclease); some of this increase is undoubtedly due to fragmented DNA, since the sonicated extracts are no longer viscous. These results therefore indicate that at least 85 percent of the cells are lysed by the freeze-thaw-lysozyme procedure.

The freeze-thaw technique described seems to have several advantages: (i) Essentially all the cells are lysed (although an appreciable proportion of the polysomes are trapped, as in other methods); (ii) the proportion of polysomes in the supernatant is high, probably partly because of the avoidance of EDTA and the low temperature—both features hinder the ribonuclease present in cell extracts from attacking the sensitive messenger RNA; (iii) the method is convenient and rapid, requiring only about 20 minutes and one centrifugation; (iv) no special equipment is needed, all operations being carried out in a single tube; (v) many tubes can be processed simultaneously; by agitation with a magnetic stirrer six tubes can be conveniently thawed at the same time in a 250-ml beaker half full of cool water; and (vi) the cells can be kept frozen, before addition of deoxycholate, to await analysis at the experimenter's convenience. Since storage at −60°C for several days did not reduce the level of polysomes in the extracts, one may perform an experiment involving many samples without having to run all the gradients immediately.

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5. Supported by NSF grant GB-1307. E.Z.R. was supported by PHS training grant GM-177-07; R.E.K., by a PHS postdoctoral fellowship.

31 May 1966

Inorganic Pyrophosphate: Formation in Bacterial Photophosphorylation

Abstract. *Inorganic pyrophosphate is identified as the major product of photophosphorylation by isolated chromatophores from* Rhodospirillum rubrum *in the absence of added nucleotides.*

Light-induced uptake of limited amounts of added orthophosphate has been demonstrated in chromatophores from the purple photosynthetic bacterium *Rhodospirillum rubrum* (*1*). Although there was evidence that part of the product formed could have been adenosine triphosphate (ATP), by phosphorylation of possibly existing endogenous adenosine diphosphate (ADP), more detailed analysis of the product(s) of the light-induced reaction was required. With a sensitive chromatographic technique (*2*) we have now demonstrated that the bulk of the reacting orthophosphate goes to pyrophosphate, and that only a minute amount occurs in other compounds. We now describe identification of the pyrophosphate and other products and discuss the possible role of pyrophosphate formation in light-induced energy transfer of the chromatophores.

Chromatophores prepared from *R. rubrum* (*1*) were illuminated in the presence of ^{32}P–inorganic orthophosphate. The incubation was stopped by 1*M* perchloric acid, and the resultant extracts were neutralized and subjected to ion-exchange chromatography; ^{32}P activity was then continuously recorded. Of two sets of experiments (Table 1), one was performed with the so-called physiological system for photophosphorylation; the other with the so-called phenazine methosulfate system (*3*). Table 1 shows only trace amounts of ^{32}P incorporated into adenosine and guanosine nucleotides; most of it is found in a nonnucleotide fraction that we identified as pyrophosphate.

Chromatograms of the chromatophore extract revealed only traces of nucleotides, on the basis of the continuous 265 mμ absorption recording of the eluate. In agreement with sensitive enzymic optical tests, the measured nucleotide content [ATP + ADP

+ AMP (adenosine monophosphate)] was at the borderline of the measuring sensitivity of the methods employed [0.02 μM/OD 880 mμ (concentration divided by optical density at 880 mμ)]. Identity of the pyrophosphate was confirmed thus:

1) The labeled compound in question appeared in the ion exchange–chromatography system in the same position as inorganic pyrophosphate.

2) Identity of the ^{32}P peak in the inorganic-pyrophosphate region of the ion-exchange chromatogram was established by thin-layer chromatography on cellulose. For the two different systems employed, the solvent of the first was methanol–formic acid–water 16:3:1 by volume; of the second, acetic acid–ethyl acetate–water 3:3:1 by volume.

3) The perchloric acid extract of the incubated chromatophores was treated with charcoal in acid solution; the ^{32}P-labeled compound was not retained on the charcoal. The solution was then subjected to ion-exchange chromatography (*4*). Fractions containing orthophosphate, pyrophosphate, and tripolyphosphate markers were separated from each other; all radioactivity was recovered in the inorganic orthophosphate and the inorganic pyrophosphate fractions.

A role of pyrophosphate in the formation of polyphosphate may be considered. In the isolated chromatophores, however, no significant amount of labeled polyphosphate was found (less than 10 percent of the ^{32}P incorporated into inorganic pyrophosphate) after illumination under the conditions described.

In view of the lack of nucleotides in the isolated chromatophores, the nucleotide content of whole cells of *R. rubrum* was measured. Nucleotide analysis by ion-exchange chromatography of a single preparation of *R. rubrum*, grown in the light, showed that the sum of free adenine, guanine, uridine, cytidine, and inosine nucleotides in the whole cell was only about 5 μM/OD 880 mμ (*5*). Optical density was measured in the sonicated cell suspension. Thus the total nucleotide content of the cells was of the same magnitude as the amount of pyrophosphate found in the chromatophores, which fact indicates that the inorganic pyrophosphate may have a quantitatively significant function in phosphate-transfer reactions of the photosynthetic bacteria.

The formation of inorganic pyrophosphate by the illuminated chromatophores may be explained by two alternative mechanisms: (i) The pyrophosphate is formed from ATP generated by photophosphorylation; this mechanism would require the presence of small amounts of endogenous adenine nucleotides in the chromatophore preparation; and (ii) it is formed directly (with no ATP involved) by a photophosphorylation process. Since the chromatographic assay shows a small peak of ^{32}P activity in the area where ATP appears in the chromatographic system (*2*), the first mechanism cannot be excluded. However, a recent report (*6*) that formation of pyrophosphate by chromatophores is not inhibited by oligomycin, in contrast with the photophosphorylation of added ADP (*7*), favors the second mechanism.

The ^{32}P compound now identified as pyrophosphate appears to be closely linked to hydrolyzing reactions, as it is rapidly broken down when the light is turned off (*1*). This observation is in line with experiments showing that chromatophores from *R. rubrum* contained pyrophosphatase activity that was even greater than adenosine triphosphatase activity and sensitive to a number of uncoupling agents (*8*).

Earlier reports suggest that the formation of inorganic pyrophosphate may be directly connected with respiration in the metabolism of both lower (*9*) and higher (*10*) organisms. Since inorganic pyrophosphate is known to be a by-product of numerous ATP-specific substrate-activation reactions, the results of some of these authors might be explained by assumption of substrate-activation processes. A more recent report is that inorganic pyrophosphate is formed more rapidly than ATP by respiring *Acetobacter suboxydans* (*11*) and cell-free extracts of *Escherichia coli* (*12*). Furthermore, phosphoenolpyruvate carboxykinase has been isolated from propionic acid bacteria that utilizes exclusively inorganic pyrophosphate as phosphate donor (*13*). These reports, together with these findings of ours, suggest that inorganic pyrophosphate functions in a main path of phosphate transfer in microorganisms.

Note added in proof: This suggestion is supported by recent experimental findings (by M. Baltscheffsky, Johnson Research Foundation, University of Pennsylvania) indicating that inorganic pyrophosphate can act as energy donor for an energy requiring steady-state change of *c*-type cytochrome in chromatophores from *R. rubrum*. This appears to be the first demonstration of a role for inorganic pyrophosphate as energy donor in an electron-transport phosphorylation system. The effect obtained with inorganic pyrophosphate is not inhibited by oligomycin, unlike the similar effect obtained with ATP.

Table 1. Light-induced formation of inorganic pyrophosphate in chromatophores from *R. rubrum*. The reaction mixture of the physiological system contained: 66 mM glycylglycine buffer, *p*H 7.4; 13.3 mM $MgCl_2$; 6.7 mM P_i^{32}; 1mM ascorbate; and chromatophore suspension giving a final absorbancy at 880 mμ of 16.6. The reaction mixture of the phenazine methosulfate system contained in addition $6.6 \times 10^{-6} M$ 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide and $4 \times 10^{-5} M$ phenazine methosulfate; final volume, 1.5 ml. The reaction medium was kept at 30°C for 5 minutes before the chromatophores were added; the reaction mixture was then preincubated for 2 minutes in the dark at 30°C before illumination by white light at about 20,000 lux for the times indicated. The reaction was stopped by addition of 1 ml of 1M ice-cold perchloric acid; the coagulated chromatophores were then removed by centrifugation. The resultant extract was rapidly neutralized in the cold with 3M KOH and then analyzed by ion-exchange chromatography on Dowex 1X8 (*2*). PPi, inorganic pyrophosphate; nm, not measured; GTP, guanosine triphosphate.

Compound	^{32}P (μM) incorporated/OD 880 mμ within (sec)				
	0	5	20	90	300
Physiological system					
PP_i	0	0.5	1.2	2.2	2.2
X_{II}*	0.02	.02	0.05	0.03	0.04
ATP†	0	nm	.01	.02	.04
ADP†	.003	nm	nm	nm	.004
GTP†	0	nm	nm	.002	.01
Phenazine methosulfate system					
PP_i	0.02	0.7	2.9	5.1	2.3
X_{II}	.02	nm	0.02	0.02	0.03
ATP	0	nm	nm	nm	.03
ADP	0	nm	nm	nm	.03
GTP	0	nm	nm	nm	.02

* Unknown compound appearing where X_{II} appears in the chromatographic system of (*2*).
† The labeled compounds are identified only by the position of the chromatogram where the corresponding nucleotides are known to appear.

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29 April 1966

Heat-Labile Serum Factor Required for Immunofluorescence of Polyoma Tumor Antigens

Abstract. *The immunofluorescent demonstration of polyoma tumor antigens in tumor cells requires a heat-labile serum component. With unheated hamster tumor serum, specific fluorescence was observed in polyoma-transformed hamster, mouse, and rat tumor cells. Heated serum usually gave little or no reactivity; the activity of such heated serum could be restored simply by the addition of fresh normal unheated hamster serum.*

We have reported unsuccessful attempts to demonstrate polyoma tumor antigens (T-antigens) in hamster and mouse tumor cells by fluorescent antibody (FA) techniques (*1*). By the same methods, however, T-antigens were shown to be present in normal mouse embryo cells undergoing lytic polyoma infection. We have since found that polyoma T-antigens can indeed be detected in virus-free tumor cells by the FA procedure and that the reaction requires a heat-labile serum factor. These results are reported here.

In our previous experiments, we first tested tumor serums by the complement-fixation (CF) test to be certain that they contained antibodies against polyoma T-antigens. For the CF test, therefore, the serums were heated at 56°C for 20 min to inactivate complement. The negative results with immunofluorescence had thus been obtained with such heated serums. In reinvestigating the problem, fresh unheated polyoma hamster tumor serum was tested with a polyoma hamster tumor cell line designated as Py-T-54. The indirect method (*1*) was used. The unheated tumor serum gave bright intranuclear fluorescence (Fig. 1*A*). When the same serum was heated at 56°C for 20 minutes, fluorescence was almost totally abolished (Fig. 1*B*). With certain high-titer tumor serum (CF titer of 1/512), heating did not destroy all activity, but the pattern of fluorescence was quite different from that of unheated serum. Such heated serum gave a granular, discrete type of fluorescence (Fig. 1*C*). These results clearly indicated that a heat-labile serum factor was required for optimum fluorescence of polyoma T-antigens. To determine whether the factor was present in normal hamster serum, nonreactive heated tumor serum was mixed with an equal volume of fresh unheated normal hamster serum and tested for reactivity. Results of this experiment showed that unheated normal hamster serum contained the factor which completely restored reactivity of the heated tumor serum. Serum from eight individual adult hamsters were tested in a similar manner and all possessed the factor, indicating that a normal serum component was involved in the reaction. The normal serum could be diluted 1 : 8 without loss of activity. The effect of heating on other polyoma hamster tumor serums was also tested; in every instance, heated serum gave either a low degree of fluorescence or negative results. On the other hand, bright fluorescence was consistently found with unheated serum.

A number of other polyoma tumor cell lines transformed in vitro or in vivo were examined for T-antigens by immunofluorescence with unheated tumor serum (Table 1.) All cell lines tested were positive; however, the degree and type of fluorescence differed with the various transformed cells. On a roughly quantitative scale, the Py-T-54 (hamster) and the Py-1923 (mouse) cells gave the brightest fluorescence (+++). In general, there appeared to be a correlation between the CF titer of the various polyoma tumor cell lines and the degree of fluorescence observed. The Py-3T3-13 mouse cell line (received from G. Todaro), which was positive by the CF test only at undiluted cell concentration (10 percent cell suspension) was nevertheless positive by immunofluorescence, indicating a high degree of sensitivity of the test. Also of interest is the fact that an SV40-polyoma (SV-Py), doubly transformed, hamster cell line derived in this laboratory (*2*) contained both the polyoma and SV40 T-antigens by immunofluorescent as well as by CF tests. Among the cells tested, two lines (Py-T-59 and BHK-21, TC-1) were previously reported as negative when tested with heated tumor serum (*1*);

Table 1. Cells tested for immunofluorescence with unheated hamster polyoma tumor serum.

Cells tested	Species	CF titer*	FA	Source of cells
		Polyoma transformed hamster cells		
Py-T-54	Hamster	1/8	+++	LBV†
Py-T-59	Hamster	UND‡	+	LBV
BHK-21, TC-1	Hamster	1/4	++	M. Stoker
SV-Py	Hamster	1/4	++	LBV
Py-1923	Mouse	1/8	+++	LBV
Py-89	Mouse	1/2	+	R. Ting
Py-1498	Mouse	1/8	++	R. Ting
Py-3T3-13	Mouse	UND	+	G. Todaro
Py-3049	Rat	1/4	+	R. Ting
		Non-polyoma tumor cells		
SV 40-C11	Hamster	—	—	LBV
Rous tumor	Hamster	NT§	—	G. Rabotti
		Normal primary cells		
Hamster embryo		NT	—	
Mouse embryo		NT	—	
Rat embryo		NT	—	

* All cells tested as 10 percent frozen and thawed cell suspensions. † LBV, Laboratory of Biology of Viruses, National Institutes of Health. ‡ Und, positive undiluted only. § NT, not tested.