plates fixed and stained on the 20th day of culture. All the subfractions of H promoted better growth than equal concentrations of L. Those subfractions which contained the components of highest molecular weight (G-100, excluded) were still effective inhibitors of expression of cartilage cell differentiation. Heat treatment (65°C for 10 minutes) of the G-100, excluded fraction before it was incorporated into the medium permitted the cartilage cells to express differentiated function. These cultures exhibited a slightly reduced growth rate, however (Table 2).

While the data do not rigorously exclude the possibility that H-supplemented media inhibit expression of the differentiated functions of cartilage and of retinal pigment cell by increasing the growth rate, cells with equally high average generation times (21.5 hours) may exhibit function or not, depending only on the absence or presence of the components of highest molecular weight (Table 2).

Growth-promoting factors are distributed among the high- and low-molecular-weight fractions of EE. Furthermore, a growth-promoting factor similar to that of the low-molecular-weight fraction (L) is spontaneously regenerated from the high-molecular-weight fraction (H, G-25) from which the low-molecular-weight components have previously been removed. If H (G-25) is allowed to stand for 24 hours in the refrigerator (2° to 4°C) and then refractionated on Sephadex G-25, a new L fraction as well as an H fraction are obtained (Table 2). The L fraction thus generated from H has growth stimulating properties similar to those of the original L fraction; the growth-promoting properties of the new H fraction are unchanged (*10*). Thus, part of the ability of H to promote growth may come from breakdown into or release of low-molecular-weight components from the large molecules in H. Similar suggestions have been made by Eagle and his collaborators (*18*) for the origin of the growth-promoting factors in serum. These workers have shown that the high-molecular-weight materials in serum, necessary for cellular growth (but not attachment), are broken down prior to utilization by the cells. Ultrafiltrates of digested serum can substitute completely for serum in most instances. Bound molecules may also be liberated from serum proteins and metabolized.

The inhibitory properties of EE may partly account for the dedifferentiation of primary cell cultures obtained in vitro by earlier workers (*5*, *17*). Similar inhibition of expression of differentiation is seen when cells are grown without EE but at high cell densities, at which they may get insufficient amounts of proper nutrients (*14*, *16*). The cells in these instances may generate inhibitory substances similar to those in EE. Conditioning of media containing whole EE or high concentration of H (*6*) definitely increases both plating efficiency and the percentage of differentiated clones; it does not improve L-supplemented media (Table 1). Conditioning may serve to add low-molecular-weight compounds from the large molecules in the medium. Conditioning may also inactivate or partially inactivate the high-molecular-weight, heat-labile, inhibitory factors in EE. For pigmented retina cells and cartilage cells the use of L has supplanted the use of conditioned media.

HAYDEN G. COON*
Biology Department,
Brandeis University,
Waltham, Massachusetts
ROBERT D. CAHN
Zoology Department,
University of Washington,
Seattle 98105

References and Notes

1. A. Carrel, *J. Exp. Med.* **17**, 14 (1913).
2. W. J. Rutter, N. K. Wessells, C. Grobstein, *Nat. Cancer Monagr.* **13**, 51 (1964); N. K. Wessells, *Proc. Nat. Acad. Sci. U.S.* **52**, 252 (1964); P. Sengel, *Ann. Biol.* **34**, 29 (1958).
3. A. H. Ebeling, *Compt. Rend. Soc. Biol.* **90**, 562 (1924).
4. L. Doljanski, *ibid.* **105**, 343 (1930).
5. S. R. Hilfer, *Develop. Biol.* **4**, 1 (1962); J. R. Whittaker, *ibid.* **8**, 99 (1963).
6. I. R. Konigsberg, *Science* **140**, 1273 (1963).
7. J. H. Hanks and R. E. Wallace, *Proc. Soc. Exp. Biol. Med.* **71**, 196 (1949).
8. Nutritional Biochemicals, Type III, 0.25 mg/100 ml.
9. J. C. Bryant, W. R. Earle, E. V. Peppers, *J. Nat. Cancer Inst.* **14**, 189 (1953).
10. Plating efficiency tests with sternal cartilage cells from 15-day-old chick embryos have not revealed a significant difference between L fractions obtained immediately and those taken after an incubation in the refrigerator of 24 hours.
11. Sephadex, coarse bead form from Pharmacia Fine Chemicals, New York. Fractionations have also been done on columns, 4 by 50 cm. Up to 120 ml of EE were fractionated at flow rates of 4 to 6 ml/min.
12. R. G. Ham, *Exp. Cell Res.* **29**, 515 (1963).
13. Armour, Fraction V.
14. R. D. Cahn and M. B. Cahn, *Proc. Nat. Acad. Sci.* **55**, 106 (1966).
15. R. D. Cahn, H. G. Coon, M. B. Cahn, "Growth of differentiated cells: Cell culture and cloning techniques," in *Experimental Techniques of Development*, F. Wilt and N. Wessells, Eds. (Thomas Y. Crowell, New York, in press).
16. H. G. Coon, *Proc. Nat. Acad. Sci. U.S.* **55**, 66 (1966).
17. H. Holtzer, J. Abbott, J. Lash, S. Holtzer, *Proc. Nat. Acad. Sci. U.S.* **46**, 1533 (1960).
18. H. Eagle, *ibid.* **46**, 427 (1960); ———, *Science* **122**, 501 (1955); ———, *J. Exp. Med.* **102**, 37 (1955); K. A. Piez, V. I. Oyama, L. Levintow, H. Eagle, *Nature* **188**, 59 (1960).
19. Supported by NSF grants GB 2619 (awarded to HGC) and GB 1832 (awarded to RDC) and grants from the American Heart Association (65-G-102), American Cancer Society (IN-32E-12) the Hartford Foundation, and the Joseph P. Kennedy, Jr. Laboratories of Molecular Medicine. We thank E. Zwilling for the use of his laboratory facilities and M. B. Cahn for her assistance.
* Current address: Carnegie Institution of Washington, Department of Embryology, Baltimore, Maryland 21210.

13 December 1965; revised 21 March 1966

Polysomes Extracted from Escherichia coli by Freeze-Thaw-Lysozyme Lysis

Abstract. *Polysomes can be extracted from* Escherichia coli *by freezing and thawing in the presence of lysozyme, followed by treatment with sodium deoxycholate. The method is simple and convenient; the yields consistently high.*

Several methods have been used to extract intact polysomes from bacteria: pressure cells (*1*), lysis of spheroplasts (*2*), and EDTA-lysozyme (ethylenediaminetetraacetate) treatment (*3*). These procedures have disadvantages: some are tedious or elaborate, and some give low or inconstant yields in terms of either RNA extracted or the proportion of the recovered ribosomes that are present in polysomes. We now describe a new method of breaking cells to provide high yields of polysomes quickly, consistently, and with greater convenience; it depends on the fact that freezing and thawing render *Escherichia coli* cells susceptible to the action of lysozyme.

In a typical experiment 30 to 100 ml of a log-phase culture of *E. coli* W, in tryptic digest medium (5×10^8 cell/ml), was poured over an equal amount of ice and centrifuged immediately for 5 minutes at 10,000*g*. The cells were resuspended in a plastic centrifuge tube in 0.5 ml of cold buffer (0.01*M* tris hydrochloride, *p*H 7.75, and 0.015*M* magnesium acetate) containing 0.5 mg of lysozyme. The suspension was frozen in acetone and solid carbon dioxide and then thawed carefully in cool water until the last bit of ice melted. After two such cycles, lysis was completed by incubation with 0.015 ml of 10-percent sodium deoxycholate for 3 minutes at 0°C. (Further cycles of freezing and thawing did not perceptibly improve the yield of RNA.)

The released DNA and cell debris were sedimented by contrifugation for 10 minutes at 30,000g, and the supernatant was analyzed by zonal centrifu-

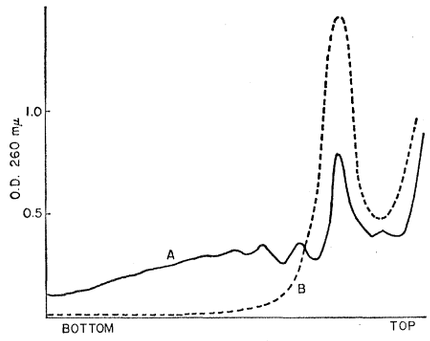

Fig. 1. Optical densities at 260 mμ. A preparation of polysomes from *E. coli* was placed on a cold, 26-ml, linear, 15- to 30-percent sucrose gradient (0.01*M* in tris hydrochloride, *p*H 7.6, and 0.01*M* in magnesium acetate) and was spun for 150 minutes at 25,000 rev/min in a Spinco-SW25 rotor at 5°C. 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.

ELIORA Z. RON
ROBERT E. KOHLER
BERNARD D. DAVIS
Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts 02115

References and Notes

1. M. Schaechter, *J. Mol. Biol.* **7**, 561 (1963).
2. Y. Kiho and A. Rich, *Proc. Nat. Acad. Sci. U.S.* **51**, 111 (1964).
3. M. Dresden and M. B. Hoagland, *Science* **149**, 647 (1965).
4. M. Dresden and E. Z. Ron, unpublished.
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