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Synthesis of Protein and Nucleic Acid by Disrupted Spheroplasts of *Pseudomonas schuylkilliensis*[1]

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Osmotically shocked spheroplasts obtained from *Pseudomonas schuylkilliensis* strain *P* contained about 54, 32, 28, and 82% of the total cellular protein, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and phospholipid, respectively. This preparation was capable of incorporating ^{32}P-orthophosphate into RNA and DNA, ^{3}H-adenosine or ^{3}H-uridine into RNA, and ^{3}H-leucine or ^{14}C-phenylalanine into protein. These activities were not found in the cytoplasmic fraction which contained most of the glucose-6-phosphate dehydrogenase activity. The synthesis of RNA by intact and disrupted spheroplast preparations was sensitive to actinomycin D, chromomycin A_3, streptovaricin, rifampin, Lubrol W, Triton X-100, and sodium deoxycholate, whereas RNA synthesis by intact cells was insensitive to these agents. Ethylenediaminetetraacetic acid, porcine pancreatic lipase, the protoplast-bursting factor, high concentrations of salts, and washing the preparation inhibited the synthesis of RNA by disrupted spheroplasts but had little or no effect on intact spheroplasts. Most of the newly synthesized RNA made by disrupted spheroplasts had the characteristics of messenger RNA. The DNA present in this preparation functioned as a template for RNA synthesis; continued protein synthesis was dependent on concomitant RNA synthesis. An unusual feature of the preparation was the finding that the synthesis of macromolecules was completely dependent on oxidative phosphorylation.

In early attempts to obtain cell-free protein- and nucleic acid-synthesizing systems from bacteria, various subcellular preparations were shown to have high activity in terms of incorporating labeled amino acids into protein (6, 30, 35, 39) or labeled nucleoside triphosphates into ribonucleic acid (RNA; 35). We obtained a subcellular preparation derived from spheroplasts from a strain of *Pseudomonas*, later classified as *Pseudomonas schuylkilliensis* (23). This preparation was able to oxidize various substrates, carry out oxidative phosphorylation, and synthesize protein and nucleic acids (28, 41). The synthesis of protein and nucleic acids in these preparations was found to be dependent on oxidative phosphorylation (28, 41). We are now interested in analyzing the relationship between the production of energy and its utilization for the synthesis of macromolecules. However, special care must be taken in any experiments with subcellular preparations derived from spheroplasts to avoid contamination with intact cells and intact spheroplasts (38).

The first part of this paper demonstrates that the RNA-snthesizing activity of our disrupted spheroplast preparation can be distinguished from that of intact cells and intact spheroplasts by differences in sensitivity to detergents, antibiotics, enzymes, and high concentrations of salts, and by differences in stability. The second part describes the conditions required for the synthesis of RNA, especially the absolute dependency on oxidative phosphorylation and the stimulation observed when amino acids and chloramphenicol were added. Lastly, the newly synthesized nucleic acids are characterized with respect to base ratios, chromatography on methylated albumin-kieselguhr (MAK) columns, and distribution into ribosome and soluble fractions.

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MATERIALS AND METHODS

Materials. ^{32}P-inorganic orthophosphate ($^{32}P_i$), ^{3}H-adenosine [T(G), 500 mCi/mmole or 4.65 Ci/mmole], ^{3}H-uridine (5-T, 22.7 Ci/mmole), and ^{3}H-DL-leucine [T(G) 102 mCi/mmole] were obtained from the Radiochemical Centre, Amersham, England. $^{32}P_i$ prepared by the Japan Atomic Energy Research Institute was also used. ^{14}C-L-phenylalanine [(U), 4.7 mCi/mmole)] was obtained from Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan.

Materials from the following commercial sources were also used: adenosine-5'-triphosphate-Na_2 (ATP, Pabst Laboratories, Milwaukee, Wis.), phosphoenolpyruvate-Na-H_2O (PEP, Boehringer Mannheim, Germany), nicotinamide adenine dinucleotide phosphate-Na_2 (NADP, Boehringer Mannheim), glucose-6-phosphate-Ba-$7H_2O$ (Calbiochem, Los Angeles, Calif.), Lubrol W (Imperial Chemical Industries, Great Britain), Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.), sodium deoxycholate (Na-DOC, Difco), Casamino Acids (Difco, vitamin-free), and Nutrient Broth (Difco). Lysozyme (EC 3.2.1.17; muramidase, twice crystallized, egg white) and deoxyribonuclease I (EC 3.1.4.5; once crystallized, bovine pancreas) were obtained from Worthington Biochemical Corp., Freehold, N.J.; lipase (EC 3.1.1.3; porcine pancreas, B grade, lot 63235 and 63672) was obtained from Calbiochem.

The following materials were generous gifts: actinomycin D (Merck Sharp and Dohme Research Laboratories, Rahway, N.J.), chromomycin A_3 (Takeda Chemical Industries Ltd., Osaka, Japan), mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan), chloramphenicol (Sankyo Co. Ltd., Tokyo, Japan), streptovaricin and rifampin (K. Nitta, National Institute of Health, Tokyo, Japan), the protoplast-bursting (PB) factor purified from pig pancreas (2; T. Yamaguchi, Toyo Jozo Co., Ohito, Japan) and highly purified lipase from *Candida cylindracea* (37; N. Tomizuka, Department of Agricultural Chemistry, University of Tokyo).

Preparation of intact cells, spheroplasts, disrupted spheroplasts, and the cytoplasmic fraction. Cells of *P. schuylkilliensis* strain *P* (23), growing in Nutrient Broth-polypeptone medium (8 g of Nutrient Broth and 5 g of polypeptone in 1 liter of deionized water, *p*H 6.8), were harvested in the exponential phase of growth, washed twice, and suspended in deionized water at a concentration of 100 mg of wet cells/ml. A portion of the cell suspension was diluted to give 20 mg of wet cells/ml in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.4) containing 5×10^{-3} M $MgCl_2$ (Mg-Tris), and this suspension was used as "intact cells." The rest of the cell suspension was treated with 3 mM ethylenediaminetetraacetic acid (EDTA) and 100 μg of lysozyme per ml in the presence of 0.5 M KCl and 0.025 M Tris-hydrochloride at *p*H 8.0. After incubation at 30 C for 30 min, a portion of the mixture was centrifuged at approximately $2,000 \times g$ for 10 min. The pellet was gently resuspended at a concentration of 20 mg of wet cell equivalent/ml in Mg-Tris containing 0.25 M sucrose. This suspension was used as "intact spheroplasts." In some experiments, the "intact spheroplasts" were taken directly from the spheroplasting mixture without sedimentation and resuspension. The rest of the spheroplasting mixture was centrifuged at $12,000 \times g$ for 5 min. The pellet was homogenized in 0.05 M Tris-hydrochloride (*p*H 7.4) containing 0.05 M $MgCl_2$ and 100 μg of deoxyribonuclease I per ml (one-thirtieth volume of the spheroplasting mixture) by use of a Potter-Elvehjem-type glass-homogenizer with a Teflon pestle. Three strokes of the pestle were made at 600 rev/min. The homogenate was diluted 10 times with 0.05 M Tris-hydrochloride (*p*H 7.4), and the suspension was further homogenized by pipetting. This homogenate, "shockate," was centrifuged at $15,000 \times g$ for 20 min. The pellet was resuspended in Mg-Tris at a concentration of 20 or 50 mg of wet cell equivalent/ml, and this suspension was used as the "disrupted spheroplasts." The supernatant obtained in this centrifugation constituted the "cytoplasmic fraction." All manipulations after the spheroplasting step were carried out at 0 to 2 C.

For determining the contents of protein, RNA, and deoxyribonucleic acid (DNA), cells or subcellular fractions were fractionated according to the modified method of Schmidt-Thannhauser and Schneider (31). The amounts of RNA and DNA in these fractions were determined from absorbance at 260 nm. Protein was determined according to the method of Lowry et al. (18). Phospholipid was analyzed on cells obtained from cultures grown in the Nutrient Broth-polypeptone medium containing $^{32}P_i$. Lipids were extracted from the lyophilized preparations with three successive portions of chloroform-methanol (2:1, v/v), and were analyzed by ascending chromatography on Amberlite WB-2 paper (Rohm and Haas Co., Philadelphia, Pa.) with diisobutylketone-acetic acid-water (8:5:1, v/v). The radioactivities of phospholipid spots were measured with a wide-area Geiger-Müller tube.

Determination of biological activities. The synthesis of RNA and protein was determined by use of a standard reaction mixture containing 0.5 ml of intact cells or subcellular preparations (derived from 10 or 25 mg of wet cells), 75 μmoles of Tris-hydrochloride (*p*H 7.4), 15 μmoles of $MgCl_2$, 2.5 μmoles of $Na_2HPO_4 \cdot 12H_2O$-KH_2PO_4 buffer (*p*H 6.7), 1 mg of Casamino Acids, and 0.1 mg of L-tryptophan and the labeled precursor. In experiments on RNA synthesis, 2 to 10 μCi of carrier-free $^{32}P_i$, 10 to 20 μCi of ^{3}H-adenosine with 0.25 μmole of carrier adenosine, or 10 to 20 μCi of ^{3}H-uridine with 0.25 μmole of carrier uridine was added; in experiments on protein synthesis, 10 μCi of ^{3}H-leucine or 0.7 μCi of ^{14}C-phenylalanine was added. The reaction was carried out in a total volume of 2.0 ml at 30 C for 10 to 40 min in an L-shaped tube with shaking. The reaction was stopped by chilling in ice and adding 0.3 ml of 40% trichloroacetic acid. RNA and protein fractions were obtained by a modified method of Schmidt-Thannhauser and Schneider (31). The radioactivity of each fraction was determined in a liquid scintillation spectrometer or by means of an end-window-type Geiger-Müller counter for single labeling experiments with $^{32}P_i$. The incorporation of $^{32}P_i$ and ^{3}H-adenosine into the internal nucleotides of RNA was measured by the following procedure. The RNA fraction (in 5% $HClO_4$) was neutralized with 8 N KOH and desalted by centrifugation. A portion of the supernatant was developed with carrier adenosine and 2',(3') nucleotide mixture by descending paper chromatography

on Whatmann 3 MM paper with *t*-butyl alcohol-99% formic acid-water (140:26.8:33.2, v/v). Adenosine (R_F = 0.45) and the mixture of nucleotides (R_F = 0.12 to 0.30, one long spot) were located by ultraviolet (UV) absorption; each spot was cut into several pieces, and the radioactivity was determined by use of a liquid scintillation spectrometer with a toluene-scintillator system (25).

Oxygen uptake was followed at 30 C by use of Warburg manometers. The reaction mixture contained, in a total volume of 2.0 ml; 1.0 ml of the disrupted spheroplasts or the cytoplasmic fraction (derived from 50 mg of wet cells), 10 μmoles of L-malate or 1 mg of Casamino Acids plus 0.1 mg of L-tryptophan, 20 μg of chloramphenicol as indicated, 75 μmoles of Tris-hydrochloride (*p*H 7.4), 15 μmoles of $MgCl_2$, and 2.5 μmoles of P_i.

Adenosine triphosphatase activity was assayed by determining the amount of P_i liberated from ATP according to the method of Marsh (20). The reaction mixture contained, in a total volume of 2.0 ml: 0.5 ml of the disrupted spheroplasts (derived from 10 mg of wet cells), 5.0 μmoles of ATP, 0.25 M sucrose as indicated, 1 mg of Casamino Acids plus 0.1 mg of L-tryptophan, 75 μmoles of Tris-hydrochloride (*p*H 7.4), and 15 μmoles of $MgCl_2$. The reaction was carried out at 30 C for 30 min.

Succinic dehydrogenase, fumarase, and glucose-6-phosphate dehydrogenase were assayed on preparations of spheroplasts, shockates, disrupted spheroplasts, or the cytoplasmic fraction, diluted to comparable concentrations. Succinic dehydrogenase was assayed manometrically by measuring O_2 uptake at 30 C in air. The reaction mixture, in a total volume of 3.0 ml, contained: 0.7 ml of the enzyme solution, 1.0 ml of 0.1 M phosphate buffer (*p*H 7.4), 0.04 μmole of cytochrome *c*, 1.2 μmoles of $CaCl_2$, 1.2 μmoles of $AlCl_3$, and 150 μmoles of sodium succinate. The center-well of the Warburg flask contained 0.2 ml of 10% KOH. Fumarase was assayed by measuring the increase of absorption at 240 nm (E^{240}) at 21 C with L-malate as a substrate. The reaction mixture, in a total volume of 3.0 ml, contained: 0.3 ml of the enzyme solution, 2.5 ml of 0.05 M phosphate buffer (*p*H 7.3), and 10 μmoles of L-malate. Glucose-6-phosphate dehydrogenase was determined from the increase in absorption at 340 nm (E^{340}) due to the reduction of NADP, at 21 C. The reaction mixture, in a total volume of 3.0 ml, contained: 0.4 ml of the enzyme solution pretreated with 5% toluene at 30 C for 15 min, 1.45 ml of 0.1 M Tris-hydrochloride buffer (*p*H 7.6), 10 μmoles of $MgCl_2$, 1.2 μmoles of NADP, 5.0 μmoles of glucose-6-phosphate, and 0.65 ml of distilled water, or 0.65 ml of 1 M sucrose when spheroplasts were used.

Ultrathin sections for electron microscopy. Disrupted spheroplasts suspended either in Mg-Tris or in 0.05 M Tris-hydrochloride (*p*H 7.4) were fixed with 1% OsO_4 at 5 C for 2 hr. After washing and dehydration, samples were embedded in polymethacrylate by use of 1% benzoyl peroxide as a catalyzer. Thin sections were cut at about 50 nm.

Isolation and fractionation of newly synthesized RNA and DNA. Disrupted spheroplasts were incubated for 15 min with $^{32}P_i$ having a high specific activity (75 to 120 μCi/2.5 μmoles) under the conditions described above for RNA synthesis. The ^{32}P-labeled particulate material was sedimented at 15,000 × *g* for 20 min. The pellet was ground with approximately 3 volumes of quartz sand (200 to 400 mesh) in a prechilled mortar for 3 min in the presence of a small quantity of Mg-Tris containing 0.2% sodium dodecyl sulfate (SDS). Alternatively, the pellet was resuspended in Mg-Tris and subjected to sonic oscillation (10 kc, 3 min); then 0.2% SDS was added to the sonic extract. RNA and DNA were extracted by the phenol method (15). In some instances, the sonic extract was centrifuged first at 15,000 × *g* for 20 min, and then at 105,000 × *g* for 90 min. Nucleic acids were then extracted by the phenol method from the precipitate (105P, resuspended in Mg-Tris) and the supernatant (105S) fractions. The ^{32}P-labeled nucleic acids were fractionated by chromatography on MAK columns according to the procedure of Mandell and Hershey (19). A linear concentration gradient of NaCl (0.4 to 1.0 M/400 ml) in 0.05 M phosphate buffer (*p*H 6.7) at room temperature was used with a flow speed of 50 drops (approximately 4.3 ml) per tube per 5 min. The susceptibility of fractions from each peak to alkaline hydrolysis was determined by measuring the conversion of cold 5% trichloroacetic acid-precipitable form to the soluble form after incubation with 0.3 N KOH at 37 C for 18 hr.

Determination of base composition. The alkaline hydrolysates of ^{32}P-RNA fractions obtained by MAK column chromatography were subjected to two-dimensional paper chromatography with carrier 2′,(3′) nucleotides. The following solvents were used: (i) isobutyric acid-1 N NH_4OH-0.1 M Na_2-EDTA (100:60:1.6, v/v), descending; (ii) methanol-ethyl alcohol-concentrated HCl-water (50:25:6:19, v/v), ascending. Four mononucleotide spots were located by UV absorption; each spot was cut out, and its radioactivity was determined directly with a wide-area Geiger-Müller tube. The per cent distribution of ^{32}P radioactivity into each spot was assumed to represent the base composition of newly synthesized RNA.

RESULTS

Synthesis of RNA by intact cells, spheroplasts, and disrupted spheroplasts. Equivalent quantities of intact cells, spheroplasts, and disrupted spheroplasts were compared by following the incorporation of $^{32}P_i$ and ^{3}H-adenosine into the internal nucleotides of RNA. Figure 1 shows that the disrupted spheroplast preparation had very little activity with respect to ^{32}P incorporation but retained approximately 50% of the ^{3}H-adenosine-incorporating activity when compared with intact cells or spheroplasts. It should be noted that only 3.4 to 4.6% of the ^{3}H-adenosine incorporation by the disrupted spheroplast preparation was located in the 3′-OH position.

A typical disrupted spheroplast preparation contained 53.6, 31.9, 28.3, and 82.4% of the total cellular protein, RNA, DNA, and phospholipid, respectively. Thus, if the initial rates of incorporation are compared on a protein basis, it is seen that the disrupted spheroplasts apparently re-

tained approximately 80% of the activity shown by intact cells for ^{3}H-adenosine incorporation but lost approximately 90% of the $^{32}P_i$-incorporating ability.

The number of viable, osmotically insensitive cells which could be detected in the disrupted spheroplast preparations was 10^{-5} to 10^{-7} of that in the corresponding intact cell suspension. However, we could not determine the total number of viable cells including viable, osmotically sensitive cells by the usual soft-agar method (38), because *P. schuylkilliensis* strain *P* grows very poorly in hypertonic media. Only 20% of the intact cells plated on SP-2 soft agar (38) containing 0.5 M KCl or 0.5 M sucrose produced colonies after 3 days of incubation. Therefore, we attempted to distinguish intact cells, spheroplasts, and disrupted spheroplasts by determining the effect of various inhibitors and treatments on the ability of these preparations to catalyze RNA synthesis (Table 1).

To test the activity of each system under the same conditions, 0.25 M sucrose, which was the most effective among several osmotic stabilizers for maintaining the RNA-synthesizing activity of spheroplasts, was added to each system even though it partially inhibited (30 to 50% inhibition) the activity of disrupted spheroplasts. The properties of intact cells were quite different from those of intact or disrupted spheroplasts. As shown in Table 1, low concentrations of detergents (Lubrol W, Triton X-100, and Na-DOC) inhibited the synthesis of RNA by spheroplasts and disrupted spheroplasts almost completely, whereas synthesis by intact cells was not affected. The synthesis of RNA by intact cells was also less susceptible to inhibition by antibiotics which effectively inhibit DNA-dependent RNA synthesis in gram-positive bacteria (10, 12, 14, 22, 26, 27). Low concentrations of actinomycin D, chromomycin A_3, streptovaricin, and rifampin had little effect on the synthesis of RNA by intact cells but inhibited synthesis by intact and disrupted spheroplasts.

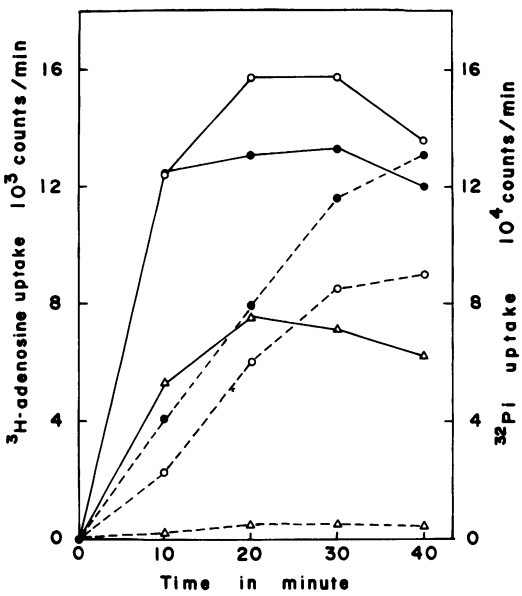

FIG. 1. *Incorporation of ^{3}H-adenosine (solid lines) and $^{32}P_i$ (dashed lines) into the internal nucleotides of RNA by intact cells (●), spheroplasts (○), and disrupted spheroplasts (△). Each reaction tube contained 8.14 × 10^6 counts/min (0.25 μmole) of ^{3}H-adenosine, 6.55 × 10^6 counts/min (2.88 μmoles) of $^{32}P_i$, and the preparation derived from 10 mg of wet cells. The protein contents of these preparations were: intact cells, 940 μg; spheroplasts, 630 μg; disrupted spheroplasts, 400 μg. Other conditions are described in Materials and Methods.*

TABLE 1. *Effects of several inhibitors or treatments on RNA synthesis by intact cells, spheroplasts, and disrupted spheroplasts*

Reaction[a]	Inhibitor or treatment	RNA synthesis[b] by		
		Intact cells	Spheroplasts	Disrupted spheroplasts
$^{32}P_i$ → RNA	None	100	100	100
	Lubrol W, 0.01%	116	0.05	0.7
	Triton X-100, 0.01%	79.2	0.02	1.1
	Na-DOC, 0.01%	92.4	0	0
	KCl, 0.1 M		90.0	67.0
	NaCl, 0.1 M		85.2	51.0
	NH_4Cl, 0.1 M		75.7	18.7
	LiCl, 0.1 M		87.7	63.4
	EDTA, 0.01 M	106	115	67.0
	Washed with			
	Nonradioactive reaction mixture		112	14.3
	Sucrose, 0.25 M		87.1	19.0
	Tris, 0.05 M; $MgCl_2$, 0.005 M; sucrose, 0.25 M		81.6	13.8
^{3}H-adenosine → RNA	None	100	100	100
	Actinomycin D, 5 μg/ml	89.7		6.6
	Chromomycin A_3, 10 μg/ml	97.5	25.2	14.3
	Streptovaricin, 1 μg/ml	89.4	20.2	38.8
	Rifampin, 1 μg/ml	100	17.7	28.0
	Lipase (porcine pancreas), 50 μg/ml		97.3	52.0
	PB factor, 25 μg/ml	100	86.0	42.0

[a] At 30 C, 15 or 30 min, shaking.

[b] Expressed as a percentage of the control. The results shown in this table are a summary of 11 different experiments. In each experiment, the results were expressed as specific radioactivity: counts per minute per milligram of RNA for $^{32}P_i$-incorporation, disintegrations per minute per milligram of RNA for ^{3}H-adenosine incorporation. Control (100%) value range: in $^{32}P_i$-incorporation experiments (counts per min per mg of RNA), 80,300 for intact cells, 50,500 to 175,500 for spheroplasts, 21,700 to 60,600 for disrupted spheroplasts; in ^{3}H-adenosine-incorporation experiments (disintegrations per min per mg of RNA), 355,000 to 742,000 for intact cells, 541,000 to 852,000 for spheroplasts, 452,000 to 950,000 for disrupted spheroplasts. The RNA contents (milligrams per reaction mixture) were: 0.402 to 0.926 for intact cells, 0.290 to 0.650 for spheroplasts, 0.129 to 0.231 for disrupted spheroplasts. Counting efficiencies of ^{3}H were 13.7 to 17.2%.

Intact and disrupted spheroplasts could be distinguished from each other by virtue of the effects of high salt concentrations and EDTA, the effect of enzymes, and stability to washing. Table 1 indicates that the synthesis of RNA by disrupted spheroplasts was slightly more sensitive to high salt concentrations and EDTA than was the synthesis catalyzed by intact spheroplasts. A much clearer difference was found in the response to washing (Table 1). Most of the RNA-synthesizing ability of disrupted spheroplasts was lost when the preparation was washed once under any one of three conditions [with the reaction mixture used for RNA synthesis, with 0.25 M sucrose, or with 0.05 M Tris-hydrochloride (*p*H 7.4) containing 5×10^{-3} M $MgCl_2$ and 0.25 M sucrose]. On the other hand, the activity of intact spheroplasts was unchanged or reduced only slightly by these procedures. In addition, the synthesis of RNA by disrupted spheroplasts, but not by intact cells or intact spheroplasts, was sensitive to two enzyme preparations: a porcine pancreatic lipase preparation and the PB factor.

The effect of increasing concentrations of these two inhibitors on the incorporation of ^{3}H-adenosine into RNA by the three preparations is shown in Fig. 2. The pancreatic lipase preparation seemed to contain other factors which produced a slight stimulation in the synthesis of RNA by spheroplasts and intact cells (Fig. 2B). The inhibitory action of the pancreatic lipase appears to be due to a partially heat-stable, nondialyzable component which is excluded from Sephadex G-25 (Table 2, experiment 1). The PB factor was also heat-stable (Table 2, experiment 2) but was inactivated by autoclaving. In contrast, a purified lipase from *C. cylindracea* did not inhibit the RNA-synthesizing ability of the disrupted spheroplasts. As shown in Table 2 (experiment 3), addition of this enzyme stimulated incorporation. Detailed studies on the effects of the lipase and PB factor will be reported elsewhere.

A comparison of the cytoplasmic fraction and disrupted spheroplasts (Table 3) shows that the latter contained most of the enzymatic machinery involved in O_2 uptake, RNA synthesis, and protein synthesis. Supporting evidence came from studies on enzyme localization. As shown in Table 4, most of the succinate-oxidizing activity was found in the disrupted spheroplasts, but a significant part of the fumarase activity and most of the glucose-6-phosphate dehydrogenase activity were found in the cytoplasmic fraction.

Requirements for RNA and protein synthesis by disrupted spheroplasts. Experiments I, II, and III of Table 5 show that RNA synthesis by disrupted spheroplasts required an oxidizable substrate such as Casamino Acids supplemented with tryptophan or a complete mixture of amino acids. KCN and anaerobiosis were inhibitory, and addition of either ATP or PEP did not overcome the inhibition caused by either KCN or anaerobiosis.

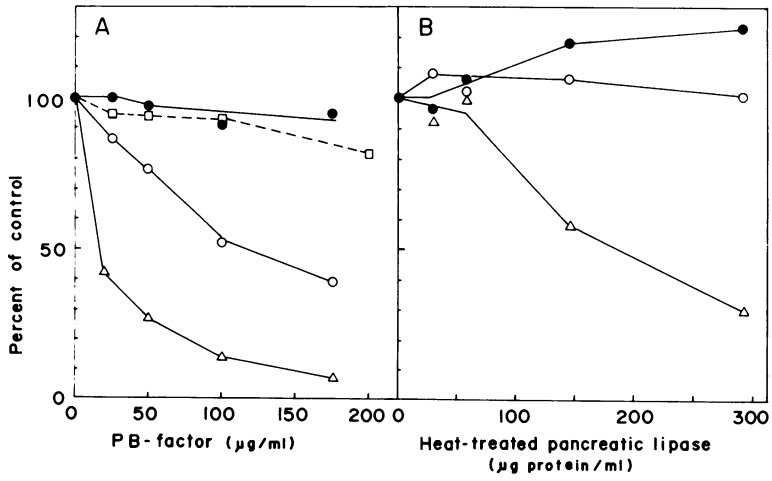

FIG. 2. *Effects of protoplast-bursting (PB) factor (A) and heat-treated (100 C, 10 min) pancreatic lipase preparation (B) on the incorporation of ^{3}H-adenosine into RNA. Symbols:* ●, *intact cells;* □, *intact spheroplasts (see Materials and Methods);* ○, *sedimented and resuspended intact spheroplasts (see Materials and Methods);* △, *disrupted spheroplasts. The reaction time was 30 min. All the reaction mixtures contained 0.25 M sucrose. The incorporation by intact spheroplasts could be distinguished clearly from that by intact cells from their sensitivity to 0.01% Lubrol-W and 25 µg of chromomycin A_3 per ml (100% and 85% inhibition, respectively); the incorporation by intact cells was not affected.*

Overall, these data indicate that endogenously generated ATP was readily used for RNA synthesis and that this endogenous source of energy could not be replaced by exogenously added ATP. Experiments IV and V of Table 5 demonstrate that exogenously added bases or nucleosides were not required, and incorporation of ^{3}H-adenosine was only partially dependent on added P_i. These results, as well as the observation that 13% of the RNA initially present in the disrupted spheroplast preparation was lost after 40 min of incubation, suggest the possibility of turnover of RNA.

TABLE 2. *Effects of pancreatic lipase and protoplast-bursting (PB) factor on RNA synthesis by disrupted spheroplasts*

Expt	Addition	Incorporation of ^{3}H-adenosine into RNA[a] (counts/min)
1	None	55,600
	Pig pancreas lipase, 50 μg/ml	12,100
	Treated lipase (80 C, 15 min), 50 μg/ml	26,600
	Dialyzed lipase, 50 μg/ml	15,800
	Lipase excluded from Sephadex G-25, 50 μg/ml (equivalent)	13,000
2	None	17,300
	PB factor, 50 μg/ml	990
	Treated PB factor (100 C, 30 min), 50 μg/ml	3,000
	Autoclaved PB factor (1 kg/cm², 120 C, 20 min), 50 μg/ml	20,000
3	None	28,000
	Pig pancreas lipase, 50 μg/ml	14,000
	Candida cylindracea lipase, 100 μg/ml	43,900

[a] Each reaction tube contained disrupted spheroplasts derived from 10 mg of wet cells. The incorporation reaction was carried out at 30 C with shaking for 30 min (expt 1 and 3) and for 15 min (expt 2). In expt 2, ^{3}H-adenosine was added after 10 min of preincubation (at 30 C with shaking).

TABLE 3. *Comparison of disrupted spheroplasts and the cytoplasmic fraction with respect to oxygen uptake, the synthesis of RNA, and the synthesis of protein*

Prepn	O_2 uptake[a]	Incorporation of $^{32}P_i$ into RNA fraction[b]		Incorporation of ^{3}H-leucine into protein fraction[b]	
		Amt of RNA	Specific activity	Amt of protein	Specific activity
Disrupted spheroplasts	16.5	529	84	856	276
Cytoplasmic fraction	2.8	916	1	1,131	0

[a] Expressed as microliters per 15 min per milligram of protein. The substrate was 1 mg of Casamino Acids plus 0.1 mg of L-tryptophan.

[b] The reaction time was 15 min. The amounts of RNA and protein are expressed as micrograms per reaction mixture. Specific activity is expressed as counts per minute per microgram (incorporation of $^{32}P_i$ into the RNA fraction) or disintegrations per minute per microgram (incorporation of ^{3}H-leucine into the protein fraction).

TABLE 4. *Distribution of enzymatic activities in subcellular fractions*[a]

Prepn[b]	Succinic dehydrogenase	Fumarase	Glucose-6-phosphate dehydrogenase[c]
Spheroplasts			16.9
"Shockate"	86.7	50.0	
Disrupted spheroplasts	131.3	55.7	10.2
Cytoplasmic fraction	23.3	32.9	34.4

[a] Activities of succinic dehydrogenase were calculated from O_2 uptake (μliters/hr), activities of fumarase were calculated from E^{240}/min with 2.1×10^3 M^{-1} cm^{-1} as the molar extinction coefficient (ϵ) of fumarate, and activities of glucose-6-phosphate dehydrogenase were calculated from E^{340}/min with 6.2×10^3 M^{-1} cm^{-1} as the ϵ of reduced NADP. Results are expressed as nanomoles of substrate converted per minute per milligram of protein.

[b] Protein content (mg/ml) of preparations used for the succinic dehydrogenase and fumarase assays; shockate, 4.18; disrupted spheroplasts, 2.38; cytoplasmic fraction, 2.15. For glucose-6-phosphate dehydrogenase assays: spheroplasts, 2.38; disrupted spheroplasts, 0.96; cytoplasmic fraction, 1.28.

[c] Each fraction was pretreated with 5% toluene at 30 C for 15 min.

The same dependence on an internal source of energy was also found for protein synthesis. Table 6 shows that the chloramphenicol-sensitive incorporation of ^{3}H-leucine proceeded at a good rate in the presence of an oxidizable substrate but that externally added ATP was ineffective in reversing the inhibition by KCN or anaerobiosis. The ineffectiveness of ATP in these experiments was not due to hydrolysis of ATP, since only 0.2 μmole (in the aerated reaction mixture, Table 6) and 0.50 μmole (in the nonaerated reaction mixture, Table 6) of P_i were found when the preparations were incubated with 5.0 μmoles of ATP for 30 min.

The stimulation in RNA synthesis observed in the presence of amino acids was not likely due to an increase in oxygen uptake nor to a concomitant increase in protein synthesis. Table 7 shows that malate, which was oxidized as well as the amino acid mixture, was only one-fourth as efficient as the amino acid mixture in supporting RNA synthesis. Furthermore, the effect of amino acids was also observed in the presence of chloramphenicol. Addition of this inhibitor produced a 1.2- to 2.2-fold increase in RNA synthesis, but

TABLE 5. *Requirements for the synthesis of RNA by disrupted spheroplasts*

Expt	Labeled precursor	Conditions[a]	Radioactivity incorporated into RNA fraction (counts/min)	Per cent of control
I	^{3}H-adenosine	1. Complete	55,600	100
		2. − oxidizable substrate	9,100	16.4
		3. + ATP (5 μmoles)	67,300	121
		4. + KCN (10^{-3} M)	19,400	34.9
		5. + KCN (10^{-3} M) + ATP (5 μmoles)	13,000	23.4
		6. Standing	16,200	29.1
		7. Standing + ATP (5 μmoles)	9,400	16.9
II	^{3}H-uridine	1. Complete	103,500	100
		2. + KCN (5×10^{-3} M)	530	0.5
		3. + KCN (5×10^{-3} M) + ATP (10 μmoles)	480	0.5
		4. + KCN (5×10^{-3} M) + phosphoenolpyruvate (10 μmoles)	440	0.4
III	^{3}H-uridine	1. − Oxidizable substrate	2,100	2.0
		2. − Oxidizable substrate + Casamino Acids + L-tryptophan	103,500	100
		3. − Oxidizable substrate + amino acid mixture	106,400	103
IV	$^{32}P_i$	1. Complete	10,600	100
		2. + Four bases (0.4 μmole each)	9,900	93.4
		3. + Four nucleosides (0.4 μmole each)	9,800	92.5
V	^{3}H-adenosine	1. Complete	65,400	100
		2. − Amino acid mixture	4,400	6.7
		3. − P_i	41,000	62.7

[a] Casamino Acids (1.0 mg per tube) and L-tryptophan (0.1 mg per tube) were added as oxidizable substrates in experiments I, II, III-2, and IV, and a mixture of 20 L-amino acids (0.6 μmole each) was added for experiments III-3 and V. In experiment IV, the four bases were adenine, guanine, uracil, and cytosine, and the four nucleosides were adenosine, guanosine, uridine, and cytidine. The reaction was carried out at 30 C with shaking (except experiments I-6 and 7) for 30 min (experiments I and V) or for 15 min (experiments II, III, and IV). In experiments II and III, the labeled precursor was added after 10 min of preincubation (at 30 C, shaking).

had no effect on O_2 uptake (Table 7). Thus, the synthesis of RNA by disrupted spheroplasts apparently did not require the concomitant synthesis of protein.

Examination of the effect of inhibitors on the incorporation of phenylalanine into protein revealed a different situation. Actinomycin D inhibited not only RNA synthesis but also protein synthesis (Table 8), indicating that most of the amino acid incorporation was dependent on transcription of DNA. The actinomycin-insensitive incorporation of ^{14}C-phenylalanine probably reflected incorporation which used messenger RNA

TABLE 6. *Incorporation of ^{3}H-leucine into protein by disrupted spheroplasts*

Expt	Addition or conditions	Radioactivity in protein fraction	
		Disintegrations/min	Per cent of control
1	No additions	31,400	100
	Chloramphenicol (40 μg)	4,100	13.1
	ATP (5 μmoles)	39,700	126
	KCN (10^{-3} M)	2,000	6.4
	KCN (10^{-3} M) + ATP (5 μmoles)	2,500	8.0
2	Aerated	42,000	100
	Nonaerated	15,200	36.2
	Nonaerated + ATP (5 μmoles)	19,900	47.4

TABLE 7. *Comparison of L-malate and Casamino Acids as energy sources for the incorporation of $^{32}P_i$ into RNA by disrupted spheroplasts*

Oxidizable substrate	O_2 uptake[a]	Radioactivity in RNA fraction[b]		
		− Chloramphenicol (A)	+ Chloramphenicol (B)	(B)/(A)
None	4.5	340	260	
L-Malate	77.2	2,120	2,500	1.18
Casamino Acids + L-tryptophan	78.3 (81.5)[c]	8,370	13,520	1.62
L-Malate + Casamino Acids + L-tryptophan	146.2	7,300	16,170	2.22

[a] Expressed as microliters per 20 min.

[b] The reaction time was 15 min. The RNA fraction was treated with activated charcoal and the radioactivity (counts per minute) adsorbed to the latter was determined. The concentration of chloramphenicol was 10 μg/ml.

[c] O_2 uptake in the presence of chloramphenicol (10 μg/ml).

TABLE 8. *Effect of actinomycin D and deoxyribonuclease on the synthesis of RNA and protein by disrupted spheroplasts*

Expt	Conditions[a]	Incorporation of ^{3}H-adenosine into RNA fraction		Incorporation of ^{14}C-L-phenylalanine into protein fraction	
		Counts/min	Per cent of control	Counts/min	Per cent of control
1	Complete	26,300	100	5,200	100
	+ Chloramphenicol, 20 μg/ml	29,000	110	700	13.5
	+ Actinomycin D, 10 μg/ml	700	2.7	1,200	23.1
2	Complete	28,200	100	4,800	100
	+ Deoxyribonuclease				
	10 μg/ml	27,200	96.5	5,200	108
	100 μg/ml	26,800	95.0	4,700	97.9

[a] Casamino Acids (1.0 mg) and L-tryptophan (0.1 mg) were used as oxidizable substrates. The reaction time was 15 min. In experiment 2, amounts of DNA (μg/reaction mixture) after 15 min of reaction were: 47 for complete, 32 for 10 μg and 30 for 100 μg of deoxyribonuclease per ml.

(mRNA) already present in the preparation at the time of addition of this antibiotic. The concentration of actinomycin used in this experiment did not inhibit the oxidation of amino acids but, in fact, stimulated oxidation approximately 1.3 times (data not shown). The interesting observation was made that both transcription and protein synthesis were insensitive to deoxyribonuclease (Table 8). The preparation was apparently permeable to this enzyme, because there was a 30% decrease in the total amount of DNA in the preparation after deoxyribonuclease addition (Table 8, footnote *a*). It will be recalled that a brief exposure to deoxyribonuclease was used in the preparation of the disrupted spheroplasts and that the preparation contained approximately 28% of the total DNA of the cell. Thus, it appears that although some DNA was hydrolyzed, the DNA which was being transcribed in the disrupted spheroplast preparation was somehow inaccessible to exogenously added deoxyribonuclease.

Effect of Mg^{2+} on function and structure. We reported earlier (41) that much of the protein-synthesizing activity of the disrupted spheroplast preparation was lost if the preparation was suspended in a Mg^{2+}-free buffer. The presence of Mg^{2+} during spheroplast disruption was also required for obtaining a preparation capable of good incorporation of $^{32}P_i$ into nucleic acids. Table 9 shows that substitution of Ca^{2+} or Mn^{2+} for Mg^{2+} during this step yielded a preparation with a reduced ability to incorporate $^{32}P_i$ into nucleic acids. In the absence of any metal ion, the "shockate" was very viscous (probably because the deoxyribonuclease present in the disruption medium could not function) and could not be separated by centrifugation (15,000 × *g*, 20 min). Thus, the entire "shockate" was used for the reaction in these experiments, and this is probably the reason for the higher content of nucleic acid shown for the fourth preparation in Table 9. However, no incorporating activity could be detected even though incorporation was measured in the presence of Mg^{2+}.

A single washing of the disrupted spheroplast preparation with 0.05 M Tris-hydrochloride buffer (*p*H 7.5) released about 35% of the protein, 50% of the RNA, 43% of the DNA, and 68% of the newly synthesized RNA (Table 10). Smaller quantities of these components were released if the disrupted spheroplast preparation was washed with 0.05 M Tris-hydrochloride buffer (*p*H 7.5) containing 5×10^{-3} M $MgCl_2$. In contrast, these treatments released very little or no phospholipids. Figure 3 shows electron micrographs of ultrathin sections of a disrupted spheroplast preparation. In the presence of Mg^{2+}, fragments of

TABLE 9. *Effect of divalent cations on the preparation of disrupted spheroplasts*

Expt	Divalent metal ion added during lysis of spheroplasts[a]	Incorporation of $^{32}P_i$ into nucleic acid by disrupted spheroplasts[b]	
		Radioactivity in total nucleic acid fraction (counts/min)	Amt of total nucleic acid (mg)
1	Mg^{2+}	2,300	0.348
2	Mn^{2+}	550	0.396
3	Ca^{2+}	950	0.366
4	—	470	0.872

[a] Metal ions were added in the chloride form at 5×10^{-3} M. In all experiments except 4, the pellet of disrupted spheroplasts was resuspended in 0.05 M Tris-hydrochloride (*p*H 7.5) containing 5×10^{-3} M $MgCl_2$. In experiment 4, the entire "shockate" was used for the incorporation reaction and 5×10^{-3} M $MgCl_2$ was added to the reaction mixture.

[b] The reaction time was 15 min. Total nucleic acid refers to material extracted with hot 5% $HClO_4$ and thus contained both RNA and DNA.

cytoplasmic membrane with attached small particulate components were observed. Some of these membranes seemed to form closed structures (Fig. 3A). Serial sections were not made and, hence, it was not possible to estimate what proportion of the membranes were present as vesicles. In the absence of Mg^{2+}, only smooth membranous structures were found (Fig. 3B).

Analysis of newly synthesized nucleic acids. Nucleic acids extracted by the SDS-phenol method from disrupted spheroplasts after 15 min of incorporation of $^{32}P_i$ were analyzed by chromatography on MAK columns (Fig. 4A). The ^{32}P-labeled nucleic acids were separated into at least five components (I'-V'). The susceptibility of fractions from each peak to alkaline hydrolysis was as follows: I', 97%; II', 18%; III', 99%; IV', 99%; V', 99%. Treatment of the nucleic acid preparation with deoxyribonuclease showed that only peaks II and II' were hydrolyzed (Fig. 4B). These data suggested that peak II' represented DNA newly synthesized by disrupted spheroplasts. This conclusion was further supported by studies on the effect of mitomycin C, known to inhibit specifically DNA replication (32). When the incorporation was carried out in the presence of 10 μg of mitomycin C/ml, the synthesis of peak II' was strongly inhibited, although some decrease in the synthesis of the RNA components was also noted. The following per cent inhibitions were found in each components: I', 10.9%; II', 76.0%; III', 21.4%; IV', 27.8%; and V', 30.5%.

RNA peaks III', IV', and V' did not coincide with ribosomal RNA (rRNA) on the MAK column fractionation pattern. When the ^{32}P-labeled disrupted spheroplasts were first fractionated into 105P and 105S fractions (see Materials and Methods), and RNA was extracted from each fraction, almost all of the III' and all of the IV' and V' components of newly synthesized RNA were obtained from the 105P fraction, the ribosome fraction (Fig. 5A). A small portion of the III' component was also present in the 105S fraction (Fig. 5B). The base compositions of III', IV', and V' were found to be markedly different from those of rRNA (Table 11), especially in the content of cytidylic acid and in the purine to pyrimidine ratio. The base compositions of these components resembled the base composition of DNA. These findings indicated that the newly synthesized species of RNA appearing in peaks III', IV', and V' had the characteristics of mRNA.

It is not known whether the DNA which remained in the disrupted spheroplast preparation was a specific portion of the total cellular DNA or was some part of the total DNA randomly included in the preparation. Comparison of DNA preparations from whole cells and from disrupted spheroplasts failed to show any significant differences in base composition or elution profiles from MAK columns.

A previous section showed that chloramphenicol enhanced the synthesis of RNA by disrupted spheroplasts (Table 7). MAK column chromatography revealed that synthesis of all nucleic acid species was increased in the presence of this antibiotic, although not to the same extent (Fig. 6).

The following increases in specific radioactivities were found; a 2.44-fold increase in peak I' (transfer RNA), a 1.68-fold increase in peak II' (DNA), a 1.99-fold increase in peak III'(mRNA), and 1.45-fold increase in peaks IV' and V' (mRNA).

TABLE 10. *Distribution of protein, RNA, DNA, and phospholipids in disrupted spheroplast preparations after washing*

Fraction[a]	Protein (μg)	RNA (μg)	DNA (μg)	Newly synthesized ^{32}P-RNA[b] (counts/min)	Phospholipids[c] (counts/min)
Disrupted spheroplasts before washing	1,780	513	40.1		6,400
After washing with Tris					
P	1,075 (65.0%)	234 (49.8%)	21.8 (56.9%)	11,800 (31.8%)	6,600 (99.1%)
S	580 (35.0%)	236 (50.2%)	16.5 (43.1%)	25,300 (68.2%)	60 (0.9%)
After washing with Mg-Tris					
P	1,420 (73.4%)	328 (68.5%)	26.7 (69.4%)	28,000 (83.3%)	
S	515 (26.6%)	151 (31.5%)	11.8 (30.6%)	5,600 (16.7%)	

[a] The pellet of disrupted spheroplasts, which was derived from 50 mg of wet cells, was suspended either in 0.05 M Tris-hydrochloride, *p*H 7.5 (Tris), or in 0.05 M Tris-hydrochloride, *p*H 7.5, containing 5×10^{-3} M $MgCl_2$ (Mg-Tris). The suspension was centrifuged at 15,000 $\times$ *g* for 20 min, and the resulting precipitate (P) and supernatant (S) were subjected to each analysis.

[b] After 15 min of incorporation reaction, ^{32}P-labeled disrupted spheroplasts were sedimented and then washed as described above.

[c] Disrupted spheroplasts were prepared from cells uniformly labeled with ^{32}P.

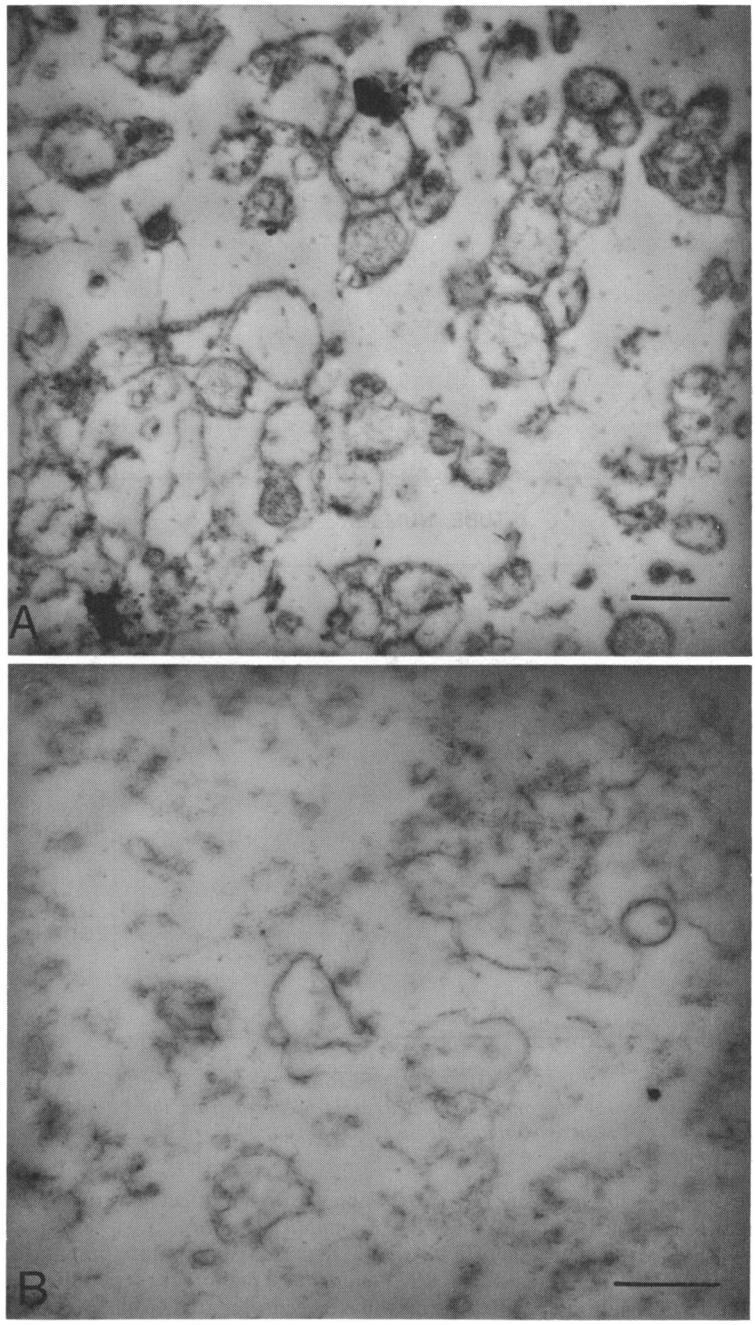

FIG. 3. *Electron micrographs of ultrathin sections of disrupted spheroplasts. The preparations were resuspended in 5 × 10^{-2} M Tris-hydrochloride (pH 7.5) containing 5 × 10^{-3} M $MgCl_2$ (A) or in 5 × 10^{-2} M Tris-hydrochloride, pH 7.5 (B). The markers represent 1.0 μm.*

DISCUSSION

The present investigation has shown that the synthesis of RNA by intact cells and two preparations of spheroplasts (intact and disrupted) can be distinguished by the effects of actinomycin D, chromomycin A_3, streptovaricin, rifampin, Lubrol W, Triton X-100, and Na-DOC. In turn, the effects of PB factor, pancreatic lipase, EDTA, salts, and differences in stability with regard to

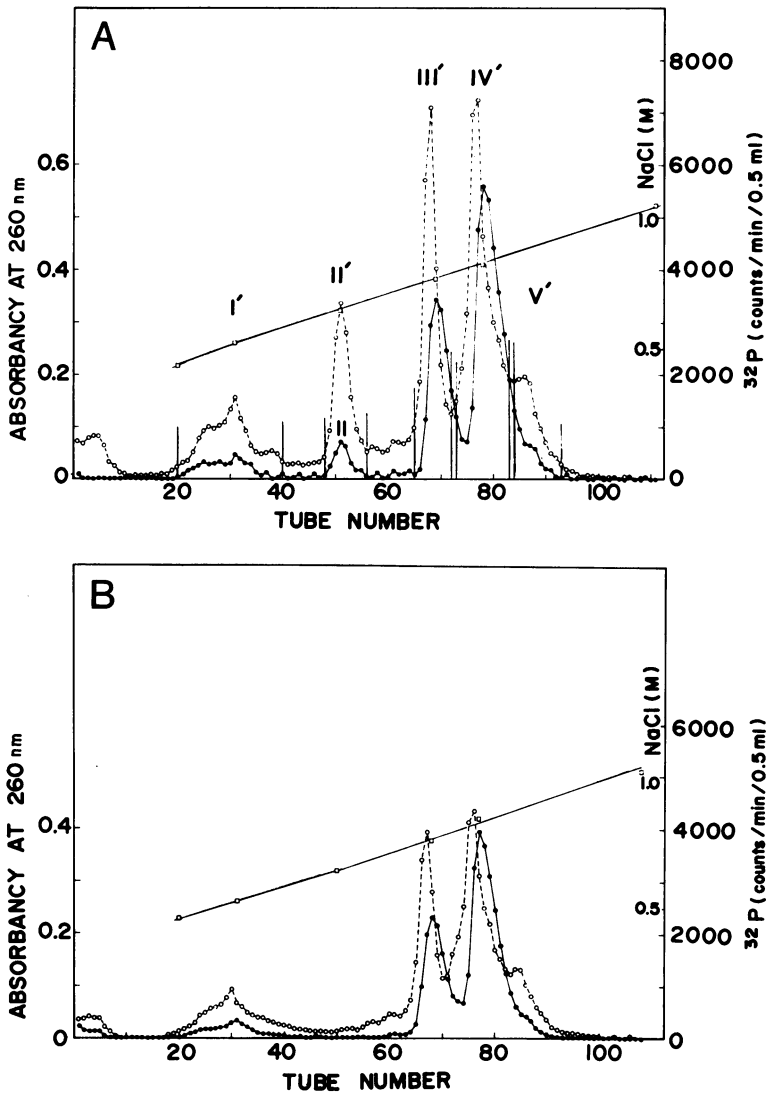

FIG. 4. *(A) MAK column chromatography of nucleic acids prepared from the ^{32}P-labeled disrupted spheroplasts. (B) After treatment of A with deoxyribonuclease I, 100 μg/ml, 30 C, 15 min. Symbols:* ●, A_{260}, ○, ^{32}P *radioactivity;* □, *NaCl concentration.*

washing serve to differentiate between the two spheroplast preparations. The ability of the PB factor to inhibit selectively the synthesis of RNA by disrupted spheroplasts is of interest. The PB factor, a protein of 13,000 daltons purified from pig pancreas, lyses the protoplast membrane of gram-positive bacteria but not gram-negative bacteria (2, 40). The effect of this factor on disrupted spheroplasts of *P. schuylkilliensis* suggests that there is a PB factor-sensitive site inside the cytoplasmic membrane of this gram-negative bacterium. Recently, we have found that the PB factor, as well as the porcine pancreatic lipase, has a phospholipase A activity which may be responsible for the effect on disrupted spheroplasts (*unpublished data*).

Disrupted spheroplast preparations capable of incorporating precursors into macromolecules have been prepared from several *Escherichia coli* and *Pseudomonas* strains but not from *Bacillus subtilis* or *B. megaterium* (21, 29). The fact that active preparations (containing fragments of the cytoplasmic membrane) were obtained only from gram-negative bacteria may be related to the ability of these bacteria to retain part of the outer membrane even after treatment with EDTA and lysozyme (3, 24). EDTA has been shown to weaken the outer membrane of *E. coli*, making it

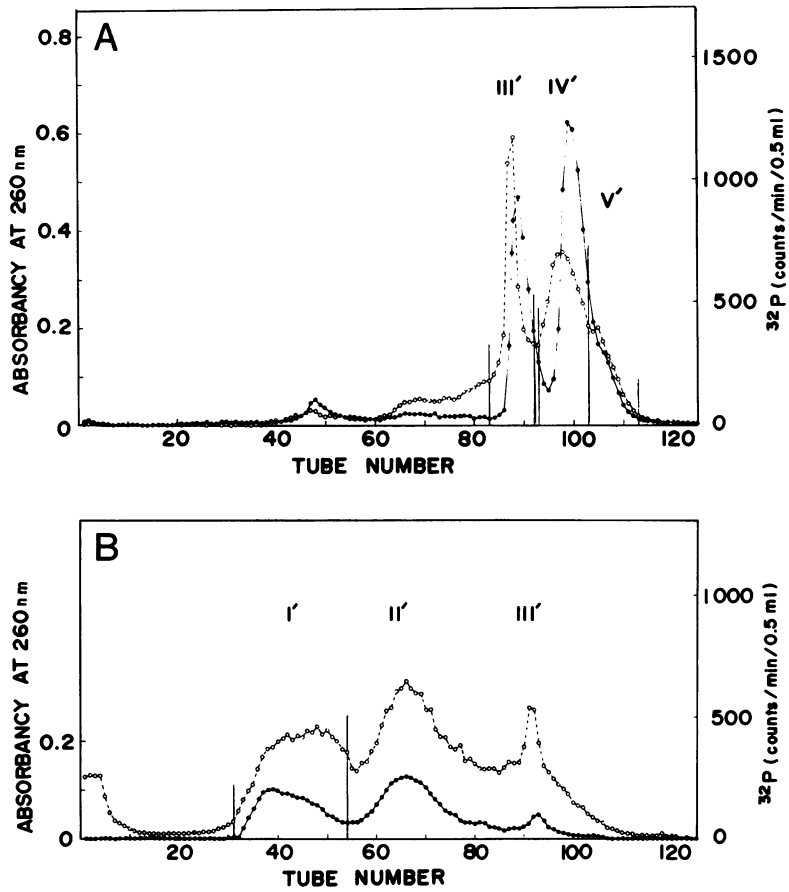

FIG. 5. *MAK column chromatography of nucleic acids prepared from the 105P fraction (A) and the 105S fraction (B), obtained from the ^{32}P-labeled disrupted spheroplasts. Symbols: ●, A_{260}; ○, ^{32}P radioactivity.*

TABLE 11. *Base compositions of newly synthesized RNA extracted from the 105P fraction*

^{32}P-RNA fraction[a]	Base composition[b] (mole %)				G + C (%)	Purine/ pyrimidine
	A	G	U	C		
III'	22.1	28.7	21.6	27.6	56.3	1.03
IV'	19.8	30.9	22.2	27.1	58.0	1.03
V'	20.5	31.1	20.2	28.2	59.3	1.07
DNA	19.5	30.4	19.5 (T)	30.6	61.0	1.00
rRNA	25.9	30.6	21.5	22.0	52.6	1.30

[a] Obtained by MAK column chromatography.
[b] A, adenine; G, guanine; U, uracil; C, cytosine; T, thymine.

more easily ruptured by osmotic pressure (3). Disruption of EDTA-lysozyme-treated spheroplasts in the presence of Mg^{2+} would be expected to stabilize the outer membrane, thereby permitting the isolation of membrane-associated structures.

Electron microscopy of the disrupted spheroplast preparations showed fragments of cytoplasmic membrane and some closed vescicles. However, the presence of large quantities of protein, RNA, and DNA indicates that the preparation had a more complex structural organization than that found in the cytoplasmic membrane per se. Mg^{2+} was required for the maintenance of this complex aggregate in a functional state. When spheroplasts were disrupted in the presence of Mn^{2+} or Ca^{2+}, the resulting preparations had a greatly reduced ability to synthesize nucleic acids, even though the nucleic acid contents of these preparations were similar to preparations made with Mg^{2+}. Removal of divalent cations by washing resulted in the release of nucleic acids and protein and in the destruction of the complex. The remaining fraction contained fragments of cytoplasmic membrane and retained almost all of the original content of phospholipids.

The most striking observation concerning the properties of the disrupted spheroplast prepara-

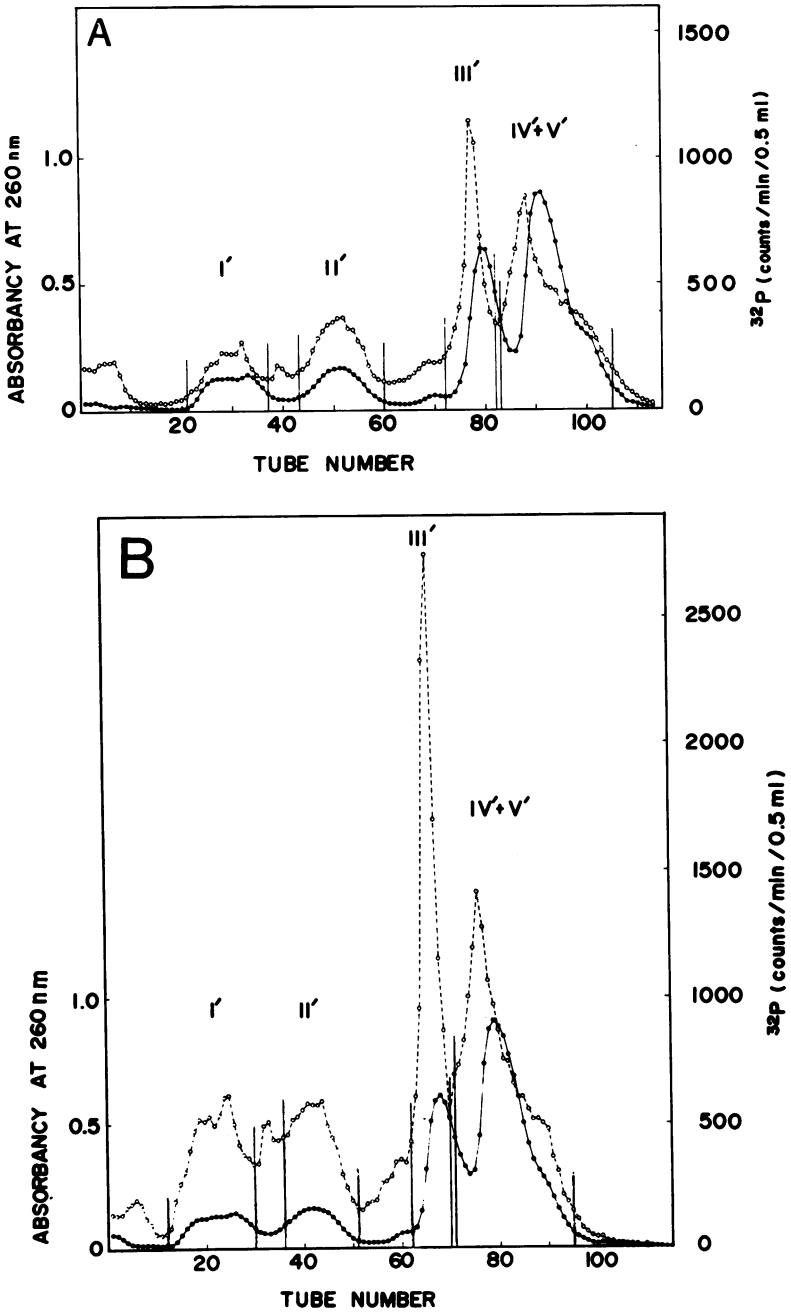

FIG. 6. *MAK column chromatography of nucleic acids synthesized in the absence (A) and in the presence (B) of chloramphenicol (20 μg/ml). Symbols: ●, A_{260}; ○, ^{32}P radioactivity.*

tion was the dependence of RNA and protein synthesis on oxidative phosphorylation and the apparently almost complete ineffectiveness of exogenously added ATP. Hydrolysis of the exogenously added ATP does not seem a likely explanation for this ineffectiveness, since the preparations had a very low adenosine triphosphatase activity. The existence of permeability barriers could provide an explanation, because closed structures resembling vesicles were observed in the electron micrographs. Possibly, such vesicles are impermeable to ATP. However, some stimu-

lation in the incorporation of amino acids (Table 6) was seen in the presence of ATP, suggesting that the preparations were not totally impermeable to nucleotides. Also, plasmolyzed preparations of another gram-negative bacterium (11) are capable of utilizing exogenously added nucleoside triphosphates for RNA synthesis.

Possibly, the nascent energy-rich compound generated during oxidative phosphorylation may not be ATP. Such high-energy intermediates have been postulated to function in ion transport and various synthetic reactions in mitochondria (1, 4, 5, 9), and in the PEP-dependent phosphotransferase mechanism of sugar transport in *E. coli* and *Staphylococcus* (17, 33). Lastly, the localization of the mechanisms for generating the energy-rich compounds in proximity to the nucleic acid-synthesizing components may be important. Thus, ATP produced at or near the cytoplasmic membrane may be utilized more efficiently for the synthesis of macromolecules than exogenous ATP. An efficient supply of energy may explain, in part, the observed association of the cytoplasmic membrane with DNA replication and transcription (7, 8, 13, 16, 36). The observation that the disrupted spheroplasts showed an increase in the synthesis of nucleic acid in the presence of chloramphenicol would be consistent with this latter explanation. It would be expected that, if protein synthesis were inhibited, increased amounts of the nascent high-energy compound produced by oxidative phosphorylation would be available for the synthesis of other macromolecules. The fact that the synthesis of all species of nucleic acid, including DNA, was enhanced in the presence of chloramphenicol supports this interpretation. Whatever the explanation may be for the observed ineffectiveness of exogenously added ATP, the present data support the conclusion that the structure and properties of the disrupted spheroplasts are different from those of the intact spheroplasts.

Studies of the incorporation of $^{32}P_i$ and ^{3}H-adenosine into nucleic acids showed that the DNA remaining in the disrupted spheroplasts could be transcribed and replicated. An unusual aspect of the disrupted spheroplast preparation was that these functions were not inhibited by deoxyribonuclease, although part of the DNA was hydrolyzed. This insensitivity with respect to deoxyribonuclease may result from the association of DNA with the cytoplasmic membrane. Snyder and Young (34) showed that genetic markers near the origin and terminus of the chromosomes of *B. subtilis* were concentrated in the membrane-bound DNA; treatment of the latter fraction with deoxyribonuclease yielded an enrichment for a marker near the origin. Although the mechanism of binding of DNA to the membrane is not known, the bound DNA may be protected against attack by exogenously added deoxyribonuclease.

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