CELL FREE SYNTHESIS OF ORNITHINE TRANSCARBAMYLASE

PALMER ROGERS* AND G. DAVID NOVELLI

*Biology Division, Oak Ridge National Laboratory,** Oak Ridge, Tenn. (U.S.A.)*

(Received February 19th, 1960)

SUMMARY

Synthesis of ornithine transcarbamylase activity by four different disrupted preparations of lysozyme protoplasts of *Escherichia coli* W cells was investigated. Whole protoplast contamination was found sufficient to account for all enzyme synthesis observed in water-lysed protoplasts. On the contrary, no contribution of whole protoplasts could be demonstrated for the ATP-dependent ornithine transcarbamylase synthesis observed in preparations disrupted in a Hughes press. Comparison of preparations disrupted by various methods indicated that the most active enzyme synthesis was found in protoplasts disrupted with a liquid-nitrogen-cooled Hughes press.

With such preparations, both ATP and glucose were found to be absolutely essential for the appearance of any new ornithine transcarbamylase, but the high ATP concentration requirement could be replaced by an energy-generating system. Although Mg^{++} was necessary for ornithine transcarbamylase synthesis, no additional stimulation activity was observed with added Mn^{++}. L-amino acid increased ornithine transcarbamylase synthesis in most experiments; however, it was not possible to repress ornithine transcarbamylase formation by arginine in disrupted protoplasts.

Evidence suggestive of an additional requirement of uridine triphosphate, cytidine triphosphate, and guanosine triphosphate for ornithine transcarbamylase formation was obtained with crude disrupted protoplasts. This requirement was augmented in the fractionated ornithine transcarbamylase-forming system.

The observations that polyvinylsulfate, which is a known RNase inhibitor, is a requirement for maximum ornithine transcarbamylase synthesis and added RNase inhibits the same process suggest a role of RNA in ornithine transcarbamylase synthesis.

Preliminary fractionation of the disrupted preparation indicated that the ornithine transcarbamylase-forming system consists of a particulate fraction together with a soluble fraction that can be recovered by acid precipitation.

The significance of these investigations is discussed.

Abbreviations: ATP, GTP, UTP, and CTP, triphosphates of adenosine, guanosine, uridine, and cytidine respectively; ADP, UDP, CDP, and GDP, diphosphates of the same substances; RNase, ribonuclease from bovine pancreas; FDP, fructose 1,6-diphosphate; OTC, ornithine transcarbamylase; Tris, tris(hydroxymethyl)aminomethane; RNA, ribonucleic acid; PEP, phosphoenolpyruvate; and PVS, polyvinylsulfate.

* Present address: Department of Agricultural Biochemistry, The Ohio State University, Columbus, Ohio.

** Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

INTRODUCTION

In a previous report[1] we described the requirements and peculiarities of the synthesis of OTC by whole cells and protoplasts of *Escherichia coli* W. The original observations on the repression of OTC formation by arginine and on the mechanism of enzyme repression in whole cells were made by MAAS AND GORINI[2-5]. The examination of OTC synthesis seemed particularly well suited for the study of *de novo* synthesis of a biologically active protein, since the formation of this enzyme is so easily controlled and its activity is readily measured.

There have been a number of recent observations on the energy-dependent incorporation of [^{14}C]amino acid into protein by various particulate fractions and disrupted preparations of cells and protoplasts of bacteria[6-10]. In addition, GALE AND SPIEGELMAN (see Review by SPIEGELMAN[11]), ROTH AND MAGASANIK[12], BELJANSKI AND OCHOA[13], and NOMURA *et al.*[14] have reported enzyme synthesis or increase in total protein by similar bacterial preparations. Some of these experiments have been difficult to interpret because of the possible contribution of whole cells or protoplasts in the preparations.

We have recently extended our studies on the biosynthesis of OTC to mechanically disrupted protoplasts of *E. coli*. This report describes new requirements for the appearance of OTC activity not found in cells or protoplasts. Evidence is cited against possible contamination by protoplasts as an explanation for the observed levels of synthesis of this enzyme in mechanically disrupted preparations. In addition, preliminary fractionation experiments indicate that both a particulate and a soluble fraction are necessary for OTC synthesis.

EXPERIMENTAL METHODS

Growth of cells and protoplast formation

Escherichia coli, strain W, was grown at 37° to midlog phase in a minimal salts and glucose medium containing arginine to repress OTC formation. Lysozyme and Versene were used to produce protoplasts as described previously[1], except that the medium for protoplast formation contained 0.33 *M* sucrose and 0.03 *M* Tris, pH 8.7. The conversion of cells to protoplasts was never less than 97 % complete as determined by microscopic counts and by viable-cell counts from surface-spread agar plates. Protoplasts were recovered from the lysozyme incubation by centrifugation for 10 min at 6000 × *g* at 15°. Usually about 4 g of protoplasts (wet wt.) were obtained from 8 g of log-phase cells.

Disruption of protoplasts

Water lysates of protoplasts were obtained by complete suspension of protoplast pellets in distilled water at 3°. Usually 40 ml of water was used /g of wet weight of protoplasts. After standing for 20 min at 3° and centrifugation at 6000 × *g* for 10 min to remove residual cells and some of the unlysed protoplasts, the lysed pieces and "ghosts" were recovered by centrifugation at 14,000 × *g* at 0° for 20 min. These water-lysed preparations still contained about 3 % protoplasts as determined microscopically with a Petroff-Hauser bacteria counting chamber.

Mechanically disrupted protoplasts were prepared in three ways.

With French pressure cell[15]: Each gram of protoplasts to be disrupted was suspended in 3.0 ml of 0.33 *M* sucrose containing 0.03 *M* Tris buffer at pH 8.5, at 3°. The suspension was then passed twice through the French pressure cell (American Instrument Co.) at a rate of about 6 ml/min while being maintained under a pressure of 16,000 pounds/in^2 with a Carver hydraulic press. The French cell was precooled at 2° before use. The disrupted preparations contained about 0.1 % of the original protoplasts as determined with the bacteria counting chamber.

With Hughes press at —20°: Protoplasts were suspended as described above. A stainless steel Hughes press[16] (Shandon Scientific Co., Ltd., London, England) was precooled in the deep freeze to — 20°, the protoplast suspension was pipetted into the block, the block was returned to the deep freeze for 20 min, and the protoplasts were then quickly pressed through the block with a Denbeigh No. 4 fly press. The disrupted material, which was collected as an icy slush, was melted before addition to the incubation flasks.

With Hughes press at —197°: Protoplasts were suspended as just described. The Hughes press was precooled in liquid nitrogen until boiling stopped. The suspension was added into the block, the block was again immersed in liquid nitrogen, and the protoplasts were pressed through as soon as possible, as described. The disrupted material was collected as a loose dry powder from the block and was quickly melted at 37° just before addition to incubation flasks. The efficiency of breakage with the Hughes press was greater than that with the French pressure cell. Less than 10^7 protoplasts remained per 10 mg of protein or about 0.01 % of the original protoplasts.

Incubation conditions for OTC synthesis

The standard incubation mixture used for these experiments contained the following in a total volume of 2.0 ml: 660 μmoles of sucrose; 200 μmoles of Tris–HCl buffer, pH 7.6; 5 μmoles of $MgCl_2$; 50 μmoles of KCl; 140 μmoles of glucose; 1.8 mg of polyvinylsulfate; 5 μmoles of ATP; 1 μmole each of UTP, GTP, and CTP; and 100 μg each of L-amino acids (except for Tables IV, VI, and IX where a balanced mixture of amino acids was used). The balanced mixture is representative of the average composition of *E. coli* protein[17] and contained in 2.0 ml the L forms of amino acids as follows: 6 μmoles of alanine, 4.7 μmoles of aspartic acid, 0.8 μmole of cysteine, 5.0 μmoles of glutamic acid, 3.8 μmoles of glycine, 0.48 μmole of histidine, 2.2 μmoles of isoleucine, 3.8 μmoles of leucine, 3.7 μmoles of lysine, 1.6 μmoles of methionine, 1.56 μmoles of phenylalanine, 2.2 μmoles of proline, 2.9 μmoles of serine, 2.2 μmoles of threonine, 0.48 μmole of tryptophan, 1.0 μmole of tyrosine, and 2.7 μmoles of valine.

The incubation mixtures were shaken at 37° in 25-ml Erlenmeyer flasks. An aliquot was then removed from each flask and stored at 1° during the incubation period as separate zero time controls. All flasks were stoppered, incubated for 60 min (or the time indicated), and the reaction was stopped by chilling to 0°.

Assays of OTC

OTC activity was measured as described. The increase in enzyme activity was always calculated as the difference between that observed after the incubation period and the zero time control for each flask. Both whole cells and protoplasts had to be treated with toluene or disrupted by other means to demonstrate full

activity. Pretreatment with toluene was not required to measure enzyme activity in disrupted protoplasts or the fractionated preparation. One unit of enzyme activity is defined as 1 μmole of citrulline produced in 1 h under the stated conditions.

Other procedures

Total protein was determined by the method of LOWRY *et al.*[18]. Counts of cells and protoplasts were made with a Petroff-Hauser bacteria counting chamber with a phase-contrast microscope. Cells and protoplasts are easily differentiated by this method as are protoplasts and "ghosts".

MATERIALS

PVS was synthesized according to the method of NOMURA *et al.*[14], using polyvinyl alcohol (98% hydrolyzed) from Matheson, Coleman, and Bell, Norwood, Ohio. Other materials were obtained from the indicated sources: ATP, GTP, UTP, CTP, ADP, GDP, UDP, and CDP from the Pabst Laboratories, Milwaukee, Wisc. (U.S.A.); the potassium salt of PEP was prepared from the silver barium salt obtained together with pyruvate kinase from C. F. Boehringer and Son, Tutzing, Germany; FDP and lysozyme from Nutritional Biochemicals Corporation, Cleveland, Ohio; L-amino acids from Mann Research Laboratories; 5 × recrystallized salt- and protease-free RNase (from bovine pancreas) from Sigma Chemical Co., St. Louis, Missouri; chloramphenicol from Parke, Davis and Company, Detroit, Michigan.

RESULTS AND DISCUSSION

Apparent OTC synthesis by water lysates of lysozyme protoplasts

Because of the success of other workers in demonstrating enzyme synthesis and amino incorporation into protein by water-lysed preparations of protoplasts, we turned to this type of preparation first in our study. The data of Table I seem to demonstrate cell-free synthesis of OTC in the water lysate. In this experiment $21.3 \cdot 10^8$ whole protoplasts synthesized 76.9 units of OTC or an average of 3.6 units/10^8 protoplasts. Under the same conditions, the water lysate, which contained only $5 \cdot 10^8$ protoplasts and 7 times as much total protein, synthesized 83.8 units of enzyme. The amount of enzyme synthesized was caused, at least in part, by those protoplasts

TABLE I

OTC SYNTHESIS IN WATER LYSATES OF LYSOZYME PROTOPLASTS

Incubations were at 37° for 60 min in a total volume of 2.0 ml.

	Protoplasts	*Lysate*
OTC units/ml	76.9	83.8
Protoplasts × 10^8/ml	21.3	5.0
Calculations		
OTC units/protoplast	3.6	3.6
OTC units synthesized by protoplasts	76.9	18.0
Apparent protoplast-free synthesis of OTC	None	65.8
If synthesis is caused by protoplasts; units/10^8 protoplasts	3.6	16.7

that survived lysis. The contribution these contaminating protoplasts could make was estimated from the amount of OTC synthesized by protoplasts that had not been lysed (Column 1, Table I). Thus it was calculated that $5.0 \cdot 10^8$ protoplasts would have synthesized 18.0 units of OTC. Since 83.8 units actually appeared it would be concluded that cell-free synthesis had produced 65.8 units. But this conslusion rests on the assumption that protoplasts surviving lysis had the same synthetic efficiency as whole protoplasts in the normal reaction mixture. It must be emphasized that protoplasts surviving lysis are in an environment rich in the intracellular components released by the lysed protoplasts and thus may perform more efficiently than protoplasts in a simple defined medium. To test this possibility, $2 \cdot 10^8$ more protoplasts were added to the water lysate, and the synthesis of OTC was measured. This experiment was, in fact, conducted simultaneously with the experiment described in Table I, but for clarity of discussion the data are presented in Table II. We added $2 \cdot 10^8$ fresh protoplasts to the water lysate of Table I that contained $5 \cdot 10^8$ protoplasts. Assuming the synthetic efficiency of whole protoplasts given in Table I, that is 3.6 units/10^8 protoplasts, we would have expected an increase in OTC synthesized by the water lysate from 83.8 to 91.0 if the added protoplasts were operating at the calculated efficiency. However, the observed increase was much higher; we found 130 units instead of the expected 91. Since the increased synthesis in the water lysate in this case is only attributable to the added protoplasts, the data show that protoplasts metabolizing in the presence of the water lysate of other protoplasts have a synthetic efficiency of 23.4 units/10^8 protoplasts rather than the 3.6 units exhibited by whole protoplasts in the standard incubation medium. This increased synthetic efficiency can more than account for the total enzyme synthesis observed in the water lysate, and, therefore, we must conclude that the observed enzyme synthesis is caused entirely by the contaminating protoplasts. The explanation for the observation that the $2 \cdot 10^8$ protoplasts had a synthetic efficiency of 23 as contrasted to an efficiency of 16.7 by $5 \cdot 10^8$ original contaminating protoplasts lies in the possibility that some of these protoplasts had been damaged during water lysis.

TABLE II

OTC SYNTHETIC EFFICIENCY BY PROTOPLASTS ADDED TO WATER LYSATE

Conditions were same as for Table I, except that $2 \cdot 10^8$ fresh protoplasts were added to the water lysate of Table I. See text for discussion.

	Expected	*Observed*
Protoplasts $\times 10^8$	5 + 2	5 + 2
If synthesis is cell free	(83.8 + 7.2) 91	130
Synthesis by added protoplasts	7.2	46.2
Synthetic efficiency of added protoplasts	3.6	23.4

OTC synthesis by mechanically disrupted protoplasts

To obtain more complete breakage of protoplasts under relatively mild conditions, we turned to mechanical disruption methods. Table III is a comparison of results obtained with three different methods of breaking lysozyme protoplasts. In a number of experiments when we used the French pressure cell, the synthesizing activity of the resulting disrupted material was consistently lower than that of preparations

TABLE III

OTC SYNTHESIS BY MECHANICALLY DISRUPTED PROTOPLASTS

Conditions as in Table I. The units synthesized in 90 min are average values obtained from two or more experiments in each case and normalized to OTC units/10 mg of protein

Material	*Units OTC synthesized in 90 min*	*Protoplasts present per 10 mg protein*
Protoplasts	680	$1 \cdot 10^{11}$
Disrupted by:		
French cell (3°)	3	$3 \cdot 10^{8}$
Hughes press (—20°)	6	
Hughes press (—196°)	16.5	$\sim 10^{7}$

made with the Hughes press at —20°, and the observed activity seemed to be a result of residual protoplasts. By lowering the temperature of the Hughes press block to that of liquid nitrogen, in an attempt to reduce denaturation to a minimum during the breakage process, we found that OTC formation was considerably improved in comparison to the other two methods. The number of protoplasts remaining after this procedure was so small that, even if they synthesized OTC at 10 times the normal rate, OTC would not be detected by our assay method. However, from Table II it is obvious that the amount of enzyme synthesized by disrupted protoplasts is quite small relative to that of the whole protoplasts. None of these mechanically disrupted preparations showed any increase in OTC activity in the absence of ATP, whereas this increase of enzyme in protoplasts was essentially unaffected by ATP. Because of the success with the liquid-nitrogen-cooled Hughes press, this method was used in our further investigation of the conditions and requirements for OTC formation.

Requirements for OTC synthesis

Fig. 1 shows the time course of the synthesis of OTC in disrupted protoplasts prepared in the liquid-nitrogen-cooled Hughes press. A comparison of curves 2, 3,

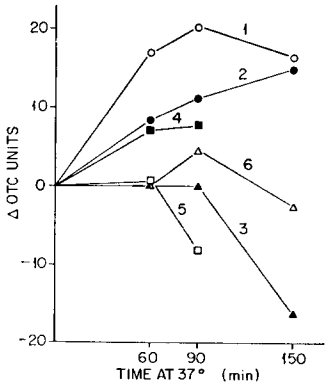

Fig. 1. The time course of OTC formation by protoplasts of *E. coli* W disrupted in a Hughes press. The ordinate is the change in OTC units measured in 2.0 ml of incubation mixture from a zero time activity of 20 units. Incubation conditions: disrupted protoplasts, 12 mg of protein. Curves: ○, complete system; ●, minus UTP, GTP, and CTP; ▲, minus UTP, GTP, CTP, and glucose; ■, nucleoside diphosphates replacing nucleoside triphosphates; □, minus ATP, GTP, CTP, and UTP; and △, complete system plus 100 μg of chloramphenicol.

and 5 indicates that the synthesis of OTC is dependent on the presence of both ATP and glucose. Curve 5 shows the results obtained when the preparation is incubated in the absence of ATP, curve 3 shows the results when the preparation is incubated in the presence of ATP but in the absence of glucose and curve 2 shows the results obtained in the presence of both glucose and ATP. The explanation for the glucose requirement is not evident to us, since, as will be shown later, glucose cannot be replaced by FDP. The glucose effect may be associated in some unknown way with the release of repression. In the absence of either glucose or ATP there was a large destruction of endogenous enzyme evident after 90 min of incubation. For this reason, we did not extend our incubations beyond 90 min in the succeeding experiments. As can be seen in curve 1, the addition of the triphosphates of guanosine, uridine, and cytidine together with ATP and glucose resulted in an increase in OTC synthesis over that with only ATP and glucose (curve 2). This stimulation of synthesis by the three nucleoside triphosphates was not consistently observed with disrupted protoplasts; and the individual contribution of each nucleotide has not been examined. Replacement of the nucleoside triphosphates by the corresponding diphosphates (curve 4) showed a level of OTC formation that is perhaps lower than that obtained with ATP alone. This indicates that the rate of generation of triphosphates by this disrupted preparation in the presence of glucose is insufficient to support maximal enzyme formation. As with cells and protoplasts[1], the appearance of OTC activity in the disrupted protoplasts is similarly inhibited by chloramphenicol, curve 6. This observation suggests that the process of protein synthesis is involved in this enzyme-forming system. None of the additions in the above experiment influenced the measurement of the activity of OTC at the concentrations present in the final reaction mixtures for enzyme assay.

Expt. I of Table IV demonstrates that Mg^{++} is required for OTC synthesis. The requirement for both ATP and glucose is also shown in this experiment. NISMAN[7] found the addition of Mn^{++} was necessary for incorporation of amino acids into protein of lysozyme lysates of *E. coli*. We found, however, that Mn^{++} added with Mg^{++} usually caused a lowering of the amount of OTC formation by disrupted protoplasts (Expt. II, Table IV).

Expt. IV of Table IV shows that it is apparently possible to substitute an energy-generating system consisting of 6.6 μmoles/ml of PEP, 5 μg of pyruvate kinase, and 0.25 μmole/ml of ATP for the tenfold higher concentration of ATP. Even under these conditions, however, the addition of glucose resulted in a marked stimulation of OTC synthesis. As noted in Expt. III of this table, it was not possible to replace the ATP and glucose requirement with FDP. Since a large increase in OTC units was not observed when the generating system was substituted for the high ATP concentration, it may be concluded that this system is not as sensitive to high ATP concentrations as had been found for other bacterial systems[6-8,11]. Fig. 2 shows an experiment in which the effect of ATP concentration on OTC synthesis was examined. This experiment was conducted in the absence of UTP, GTP, and CTP in order to rule out possible transphosphorylations to form ATP. Maximum OTC synthesis apparently takes place at about 5 μmoles/ml of ATP, or about twice the concentration used in the standard incubation mixture in these experiments.

NOMURA *et al.*[14] found that PVS was necessary for maximal appearance of amylase in lysozyme lysates of *Bacillus subtilis*. The action of PVS was attributed to

TABLE IV

REQUIREMENTS FOR OTC FORMATION

The units of enzyme activity shown are the measured increases after 60 min of incubation at 37°. "Complete" incubations contained the standard incubation mixture described in METHODS, except for Exp. I, in which no UTP, CTP, or GTP was added. In Exp. IV, the generating system consists of: 5 μg/ml of pyruvate kinase, 6.6 μmoles/ml of phosphoenolpyruvate, and 0.25 μmole/ml of ATP instead of 2.5 μmoles/ml. 10–12 mg of disrupted protoplast protein were added to each incubation mixture.

Expt.	*Incubation conditions*	*Δ units of enzyme activity/2 ml*
I	Complete	8
	Minus $MgCl_2$	0
	Minus glucose	0
	Minus ATP	0
II	Complete	13
	Minus nucleoside triphosphates	0
	Plus $MnCl_2$, 5 μmoles	7
	Plus $MnCl_2$, 10 μmoles	6
III	Complete	17
	FDP 100 μmoles instead of ATP and glucose	0
	Imidazole buffer, 100 μmoles per ml instead of Tris buffer	11
IV	Complete	17
	Generating system — no glucose	17
	Generating system + glucose	26

its inhibition of the intracellular RNase. As demonstrated by the data of Table V, we also found an enhancement of the appearance of OTC synthesis in the presence of PVS. Therefore this compound was added routinely in all experiments with disrupted protoplasts. In contrast, synthesis of OTC by whole cells or protoplasts never showed any response to the presence of PVS in the incubation medium. As shown in Table V, the RNase activity of disrupted preparations of *E. coli* was inhibited to 50% of the control level by addition of the amount of PVS found to stimulate OTC synthesis. In the absence of PVS, addition of pancreatic RNase completely blocked OTC formation,

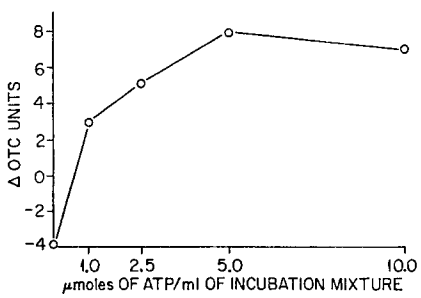

Fig. 2. ATP requirement for OTC synthesis by protoplasts disrupted in a Hughes press. The ordinate is the measured increment of enzyme units formed per 2.0 ml after 60 min of incubation at 37°. Incubation mixtures did not contain UTP, CTP, or GTP. 10 mg of disrupted protoplast protein were added to each incubation mixture.

TABLE V

EFFECT OF POLYVINYLSULFATE ON OTC SYNTHESIS AND RNASE ACTIVITY

OTC formation for whole cells and protoplasts is shown as units synthesized per 1 mg of protein. OTC formation for disrupted protoplasts is shown as units synthesized per 10 mg of protein. RNase activity was measured as release of cold perchloric acid-soluble material absorbing at 260 mμ in the Beckman DU spectrophotometer after 60-min incubation at 37° of 10 mg of disrupted protoplasts. The standard incubation medium was used, and all incubations were at 37° for 60 min in the above experiments, except that triphosphate and amino acids were omitted from the RNase-activity determinations.

Materials	*Control*	*PVS 0.9 mg/ml*	*PVS 1.8 mg/ml*
		OTC activity	
Whole cells	363	313	—
Protoplasts	68	65	—
Disrupted protoplasts	3	13	—
Disrupted protoplasts + added RNase 0.5 mg	0	7	—
		E. coli RNase activity	
Disrupted protoplasts	22.2	11.8	6.2

whereas in the presence of PVS this inhibition was only 50%. These experiments suggest that OTC synthesis is dependent on the presence of intact RNA, as was assumed from other experiments with intact protoplasts[1].

In some experiments, disrupted protoplasts were stimulated by a complete mixture of amino acids minus arginine. The experiments shown in Table VI indicate a considerable variability of this effect, but in most cases demonstrate the superiority of balanced mixture of amino acids over that of a mixture containing equal gram quantities of amino acids. This peculiarity of the amino acid requirement was also evident in the amino acid-incorporation studies with lysed lysozyme protoplasts carried out by NISMAN[7]. CONNELL *et al.*[6] also reported variable results with respect to an amino acid requirement for incorporation of radioactive amino acids into *Azotobacter* particles.

TABLE VI

AMINO ACID STIMULATION OF OTC FORMATION IN DISRUPTED PROTOPLASTS

The units of enzyme activity shown are the increases after incubation at 37° for 60 min. See METHODS for content of the balanced mixture of L-amino acids and the incubation mixture. Disrupted protoplasts: 10 mg of protein.

Conditions	*Units of enzyme activity/2 ml*		
Experiment:	*(A)*	*(B)*	*(C)*
No amino acids	3	14	3
L-amino acids without arginine, 200 μg each	6	18	4
L-amino acids without arginine, balanced mixture	12	—	7
L-amino acids, balanced mixture, with 200 μg of arginine	12	16	—

As reported previously, there is complete repression of OTC in whole cells with as little as 20 μg of arginine. On the other hand, protoplasts could not be repressed with as much as 200 μg of arginine. This was interpreted as indicating a loss of the ability to concentrate arginine by protoplasts. This interpretation has now been confirmed by MAAS[19]. Although about a 30% repression of OTC formation in whole

TABLE VII

EFFECT OF NUCLEOTIDE TRIPHOSPHATES ON OTC SYNTHESIS BY PROTOPLASTS AND DISRUPTED PROTOPLASTS

Units of enzyme are the increases observed after incubation at 37°. $4 \cdot 10^8$ protoplasts were added in (1) and (3). Disrupted protoplasts 14 mg protein.

	Δ units of enzyme activity	
	Minus nucleotides	*Plus nucleotides*
1. Protoplasts	17	13
2. Disrupted protoplasts	0	13
3. Mixture 1 + 2	22	32

protoplasts was observed, from the data in Table VI it is evident that the addition of 200 μg of arginine had no affect on the increase in OTC in mechanically disrupted protoplasts. This suggests either that arginine itself is not the repressor molecule or that even higher concentrations of arginine are necessary to effect its binding to the OTC-forming site.

Contribution of whole protoplasts to synthesis of OTC by the disrupted system

Although the small number of protoplasts remaining in the disrupted preparations seemed to preclude the probability that the increases in OTC observed were attributable to whole protoplasts, an experiment was designed to test this contingency. From the data of Tables I and II it was conceivable that a few whole protoplasts in the presence of disrupted cellular material together with glucose, ATP, UTP, CTP, and GTP could be stimulated to an extraordinary rate of enzyme synthesis. In the experiments shown in Tables VII and VIII, protoplasts, disrupted protoplasts, and a mixture of both were incubated in flasks with and without the triphosphates. $4 \cdot 10^8$ protoplasts, when incubated alone, were slightly inhibited by addition of triphosphates in this experiment (Table VII), whereas the mechanically disrupted protoplasts required the presence of these compounds for net appearance of OTC activity. Calculation from the data obtained by mixing the same quantity of protoplasts with the disrupted preparation (Table VIII) reveals that there was a definite small stimulation of OTC synthesis caused by the added protoplasts. Since the extent of the

TABLE VIII

CELL-FREE SYNTHESIS OF OTC BY DISRUPTED PROTOPLASTS

Conditions and data the same as for Table VII.

	A	*B*	*C*
	Protoplasts	*Disrupted protoplasts*	*A + B*
OTC-synthesized units	13	13	32
Protoplasts × 10^8	4.0	<0.1	4.0
Calculations			
OTC synthesis by added protoplasts	13	—	19
Units OTC/protoplasts × 10^8	3.3	—	4.8
Cell-free synthesis (corrected for protoplast contribution)	—	13	13

stimulation observed was independent of the presence of ATP and other triphosphates, it is concluded that the ATP-dependent OTC formation observed in the disrupted protoplasts cannot be the result of contamination of whole protoplasts in the preparation.

Preliminary fractionation of the OTC-forming system

The data thus far presented suggest that cell-free synthesis of OTC takes place in the disrupted protoplasts system. To reveal further requirements for synthesis, preliminary attempts were made to fractionate the OTC-forming system. Immediately after disruption of the protoplasts in the Hughes press, the crude material was centrifuged twice at 6000 × *g* for 10 min at 0° to remove large pieces and the few remaining whole protoplasts. The resulting supernatant was then centrifuged at 105,000 × *g* for 30 min at 0°. The clear supernatant was removed and the particles

TABLE IX

FRACTIONATION OF THE OTC-FORMING SYSTEM FROM DISRUPTED PROTOPLASTS

The units of enzyme activity shown are the increases after incubation at 37° for 60 min. Disrupted protoplasts, 10 mg of protein; washed particles alone, 3.5 mg of protein; pH 5 fraction, 7.5 mg of protein. See METHODS for incubation mixture and the text for fractionation procedure. Aged particles were stored for 72 h at 1°.

Material	*Δ Units of enzyme activity per 2.0 ml*		
	Control	*ATP*	*ATP, UTP, CTP, GTP*
Disrupted protoplasts	3.2	15.0	16.0
Washed particles, 105,000 × *g*	2.6	1.2	2.6
pH 5 Fraction	0	0.5	0
Particles + pH 5 fraction	—1.8	5.0	9.2
Aged particles + pH 5 fraction	—1.8	—0.6	0.4

were washed in 0.33 *M* sucrose, resedimented at 105,000 × *g*, and suspended in 0.33 *M* sucrose. The 105,000 × *g* supernatant was carefully adjusted to pH 5.1 with cold acetic acid, centrifuged at 6000 × *g* for 10 min at 0°, and the precipitate was resuspended in 0.33 *M* sucrose. The data in Table IX show the effect of this fractionation procedure on OTC formation by this system. Incubation of the washed particles alone resulted in only a small increment of OTC that was apparently independent of added ATP. The pH 5 fraction, when incubated with or without added triphosphates, showed no OTC formation. However, when particles and pH 5 fraction were incubated together, a definite ATP-dependent formation of OTC activity was observed. Further stimulation of the formation of this activity occurred with the addition of UTP, CTP, and GTP. As shown in Table IX, after these particles had been aged for 72 h at 1°, this enzyme-forming ability was completely lost. It seems evident from this fractionation experiment that both a particulate and a soluble component are required to carry out OTC formation. This result corresponds to the experience of many investigators studying protein synthesis in a number of different cellular systems[10,13,20,24]. Further experimental work will be necessary to determine if the sequence of steps, from amino acid activation through transfer RNA to final incorporation into a protein molecule by "ribosomes", studied extensively with the aid of ^{14}C amino acids, takes part in the formation of OTC observed in these experiments.

Considering all of the data reported in the foregoing experiments, a number of

points should be stressed. First, although the observed increases of OTC activity in all of the experiments with disrupted protoplasts was low, it represents between 1–2 % of the synthetic activity of the protoplast. Since increase in total protein was not consistently observed in these experiments, it is possible that the activity increases noted were attributable to a release of template-bound OTC, perhaps triggered by ATP, rather than the *de novo* synthesis of new OTC molecules during the incubation periods. Such a metabolic release of serum albumin has been observed by PETERS[25]. Second, although only the synthesis of OTC has been measured in these experiments, this does not exclude the simultaneous participation of some of the components of the incubation mixture in other reactions, such as template repair or fabrication of other materials related to the enzyme-forming machinery. Last, although the experiment demonstrating an inhibition of OTC formation by chloramphenicol suggests net protein synthesis, the measure of an increase in enzyme activity alone is not an unequivocal demonstration of protein synthesis. This observation must be further substantiated with evidence that amino acids are incorporated into the peptide sequences of this specific enzyme. Thus, in conclusion, it can be said that the evidence to date strongly suggests that a cell-free and protoplast-free system from bacteria, which can be fractionated into two parts, supports formation of OTC that is dependent on both amino acid and energy.

ACKNOWLEDGEMENT

This investigation was aided by a postdoctoral fellowship grant from the American Cancer Society.

REFERENCES

1 P. ROGERS AND G. D. NOVELLI, *Biochim. Biophys. Acta*, 33 (1959) 423.
2 L. GORINI AND W. K. MAAS, *Biochim. Biophys. Acta*, 25 (1957) 208.
3 L. GORINI AND W. K. MAAS, in W. D. MCELROY AND B. GLASS, *The Chemical Basis of Development*, Johns Hopkins Press, Inc., Baltimore, 1958, p. 469.
4 L. GORINI, *Bull. soc. chim. biol.*, 40 (1958) 1939.
5 J. H. SCHWARTZ, W. F. MAAS AND E. J. SIMON, *Biochim. Biophys. Acta*, 32 (1959) 582.
6 G. E. CONNELL, P. LEUGYEL AND R. C. WARNER, *Biochim. Biophys. Acta*, 31 (1959) 391.
7 B. NISMAN, *Biochim. Biophys. Acta*, 32 (1959) 18.
8 E. F. GALE AND J. P. FOLKES, *Biochem. J.*, 59 (1955) 661.
9 E. F. GALE, in G. TUNERALL, *Recent Progress in Microbiology*, Almquist and Wiksel, Stockholm, 1959, p. 104.
10 M. LAMBORG, unpublished data.
11 S. SPIEGELMAN, in W. D. MCELROY AND B. GLASS, *The Chemical Basis of Heredity*, Johns Hopkins Press, Inc., Baltimore, 1957, p. 232.
12 E. M. ROTH AND B. MAGASANIK, *American Chemical Society Abstracts, Division of Biological Chemistry, 1959*, Abstract No. 22.
13 M. BELJANSKI AND S. OCHOA, *Proc. Natl. Acad. Sci. U. S.*, 44 (1958) 494.
14 M. NOMURA, J. HOSODA AND S. NISHIMURA, *Biochim. Biophys. Acta*, 29 (1958) 161.
15 C. S. FRENCH AND H. W. MILNER in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press Inc., New York, 1955, p. 64.
16 D. E. HUGHES, *Brit. J. Exptl. Pathol.*, 32 (1951) 97.
17 R. B. ROBERTS, P. H. ABELSON, D. B. COWIE, E. T. BOLTON AND R. J. BRITTON, *Carnegie Inst. Wash. Publ.*, No. 607 (1955).
18 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
19 W. K. MAAS, *Biochem. Biophys. Research Communications*, 1 (1959) 13.
20 P. C. ZAMECNIK AND E. B. KELLER, *J. Biol. Chem.*, 209 (1954) 337.
21 J. L. SIMKIN AND T. S. WORK, *Biochem. J.*, 67 (1957) 617.
22 G. C. WEBSTER, *Arch. Biochem. Biophys.*, 70 (1957) 622.
23 S. B. WEISS, G. ACS AND F. LIPMANN, *Proc. Natl. Acad. Sci. U. S.*, 44 (1958) 189.
24 J. MAGER AND F. LIPMANN, *Proc. Natl. Acad. Sci. U. S.*, 44 (1958) 305.
25 T. PETERS, JR., *J. Histochem. Cytochem.*, 7 (1959) 224.