THE ACTIVATION AND INCORPORATION OF AMINO ACIDS BY SUBCELLULAR FRACTIONS OF *BACILLUS MEGATERIUM* STRAIN M*

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SUMMARY

Cell fractions obtained by the lysis of protoplasts of *B. megaterium* strain M were analysed for protein, nucleic acid and the amino-acid-activating and -incorporating enzymes. The fraction including the cytoplasmic membranes contains an average of 17% of the cell protein, 12% of the cell RNA, and 37% of the cell DNA. The amino-acid-incorporating ability of this fraction is greater than that of any other cell fraction, and the amino acids are incorporated under conditions in which no amino-acid-dependent PP exchange with ATP can be demonstrated. Essentially all of the amino-acid-activating enzymes are associated with the supernatant of a 24,000 × *g* centrifugation. The incorporating activity of the "membrane fraction" is stimulated by soluble RNA from either *B. megaterium* or *E. coli*, a mixture of ribonucleoside diphosphates, and by fragmentation of "membranes" with either digitonin or perfluorooctanoate. Incorporation into a hydroxylamine-stable form is insensitive to the action of ribonuclease and is sometimes depressed by the presence of a complete amino acid mixture. The significance of these findings is discussed.

INTRODUCTION

In recent years, there has been an increasing amount of evidence to suggest a central metabolic role for the bacterial cytoplasmic membrane. Since more than 90% of the bacterial cytochromes, succinic dehydrogenase, and other oxidative enzymes

Abbreviations used in this paper: ATP = adenosine triphosphate; ADP = adenosine diphosphate; GTP = guanosine triphosphate; PP = pyrophosphate; RNA = ribonucleic acid; DNA = deoxyribonucleic acid; RNAase = ribonuclease; S-RNA = soluble RNA; Tris = tris (hydroxymethyl)aminomethane; Medium D = 0.04 *M* Tris buffer pH 7.4 containing $2 \cdot 10^{-3}$ *M* $MgSO_4$; PFO = perfluoro-octanoate; TCA = trichloracetic acid; XDP = mixture containing equal amounts of each of the four ribonucleoside diphosphates; AA = equilibrated amino acid mixture containing L-amino acids in ratios corresponding to their occurrence in *E. coli* proteins; ala = alanine; arg = arginine; asp = aspartic; asp-NH_2 = asparagine; cys-Sh = cysteine; glu = glutamic; glu-NH_2 = glutamine; gly = glycine; his = histidine; ileu = isoleucine; leu = leucine; lys = lysine; meth = methionine; phe = phenylalanine; pro = proline; ser = serine; thr = threonine; try = tryptophan; tyr = tyrosine; val = valine.

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are found associated with the membranes of *Bacillus megaterium*[1,2] and *Staphylococcus aureus*[3], it is apparent that membranes perform many of the enzymic functions of mammalian mitochondria[4].

In addition, there is substantial evidence that the bacterial cytoplasmic membrane is the principal site for protein biosynthesis, and therefore has much in common with the liver microsome[5].

When protoplasts of either *B. megaterium*[6] or *Escherichia coli*[7] are exposed to radioactive amino acids for short periods of time and then fractionated with respect to cell components, the specific activity of the membrane proteins is found to be greater than that of other cell proteins. Furthermore, experiments with isolated cell membranes of *B. megaterium* strain KM by BUTLER *et al.*[6] have shown that of all the components of a total lysate, the membrane fraction is the most active in incorporating amino acids into protein. Similar results have been reported for membranes and membrane fragments of *E. coli* (NISMAN[8]; SPIEGELMAN[7]; NISMAN AND HIRSCH[16]) membrane fragments of *Alcaligenes faecalis* and *Azotobacter vinelandii* (BELJANSKI AND OCHOA[9]), and the membrane-containing fraction of *Staphylococcus aureus* (GALE[10]).

Experiments with liver microsomal and supernatant systems[11,12,13] have greatly influenced the current ideas on protein synthesis. It is commonly thought that the following activation reaction is the first enzymic step in protein synthesis:

$$\text{ATP} + \text{amino acid} \rightleftarrows \text{adenyl-amino acid} + \text{PP}$$

the amino acid is then transferred to low molecular weight RNA[13,14] and is then incorporated into protein in the presence of ribonucleoprotein particles and GTP[15]. The incorporation is strongly inhibited by the presence of ribonuclease. Results consistent with this scheme have been obtained with preparations from *E. coli* (SPIEGELMAN[7]; NISMAN[8] and NISMAN AND HIRSCH[16]). The washed membranes containing fraction incorporates amino acids, carries out an amino-acid-dependent PP exchange with ATP, and contains RNA. However, NISMAN[8] was unable to demonstrate an effect of GTP on incorporation.

Recently, both NISMAN AND FUKUHARA[17] and BELJANSKI AND OCHOA[9] have described bacterial amino-acid-incorporating systems, with properties different from those of the liver supernatant systems. Phenol-treated *E. coli* membrane fragments[17] and membrane fragments of *Alcaligenes faecalis*[9] incorporate amino acids, but do not catalyse an amino-acid-dependent PP exchange reaction with ATP. Nor are they able to form the complex S-RNA-AA. The activity of the *E. coli* membrane fragments is stimulated by the presence of RNA-ase[17].

The work reported in this paper was undertaken in an attempt to localize both the amino-acid-incorporating system and the amino-acid-activating systems in a lysozyme-sensitive organism, in which there would be no interference by the cell wall material. In addition, the cell fractions were analysed for protein and nucleic acid. The results show that the amino-acid-incorporating ability of the fraction containing the membranes of *B. megaterium* is greater than that of any other cell component, and that this activity is not inhibited by RNA-ase. The fraction including the membranes contains an average of 12% of the cell RNA, 37% of the cell DNA, and at most traces of amino-acid-activating enzymes.

MATERIALS AND METHODS

Preparation of enzyme fractions

We are indebted to Dr. IONESCO for a culture of *Bacillus megaterium* strain M[18]. Cells were grown with vigorous aeration at 30° on a medium consisting of 0.3% Bacto casitone, 0.3% Bacto tryptone, 0.2% Bacto peptone, 0.1% yeast extract, 0.5% K_2HPO_4, 0.05% $MgCl_2$, and 0.01% $FeSO_4 . 7H_2O$ (pH adjusted to 7.2)*. Cells were harvested by centrifugation after growth for 12–18 h, washed once with 0.04 *M* Tris buffer pH 7.4 containing $2 \cdot 10^{-3}$ *M* $MgSO_4$ (Medium D), and were finally resuspended in Medium D containing 0.25 *M* sucrose.

For the preparation of protoplasts, crystalline lysozyme was added to a final concentration of 0.4 mg per ml. Although the lysozyme to cell ratio was kept constant, there was a considerable day-to-day variation in the speed of lysozyme action. Protoplast formation was essentially complete after incubation at room temperature for from 20 to 150 minutes. Protoplasts were collected by centrifugation at 13,000 *g* for 10 minutes and were resuspended in Medium D containing 0.25 *M* sucrose.

Protoplasts were lysed at 4° either by osmotic shock or by lysis in 0.25 *M* sucrose in the presence of digitonin[16,37]. For osmotic lysis, enough Medium D was added to protoplasts suspended in hypertonic sucrose (0.25 *M*) to lower the sucrose concentration to 0.04 *M*. Lysis was complete after stirring for 5 minutes. The addition of digitonin (final concentration of 3–7 mg/ml) to protoplasts suspended in hypertonic sucrose resulted in complete lysis after 5–10 minutes of stirring. With either procedure, the $MgSO_4$ concentration of the total lysate was adjusted to 10^{-2} *M*.

All the steps involved in the fractionation of the total lysate were performed at 4°. The first low speed centrifugation (Fig. 1) separates the total lysate into a membrane (P_1)- and supernatant (S_1)-containing fractions. The ribosomes[19] (P_4) present in the S_1 fraction were obtained by centrifugation at 100,000 × *g*. The P_1 fraction was washed once and in some experiments, the resulting P_2 fraction was treated with either digitonin or the anionic detergent, perfluoro-octanoate (PFO)**. These compounds induce membrane fragmentation. The supernatant fractions (S_2, S_3, S_4, S_6) were precipitated with 2 volumes of ethanol at −20°. The resulting pellets were redissolved in Medium D and then assayed for enzymic activity.

Definition of the fractions obtained by differential centrifugation

Cell fractions obtained from osmotically shocked protoplasts by differential centrifugation can be defined essentially as follows:

(1) fraction (P_1) or (P_2) or "membranes" sedimented at 24,000 × *g* including the bulk of the cytoplasmic membranes and associated with a variable amount of DNA and RNA.

(2) fraction (S_1) which submitted to high speed centrifugation for 120 minutes or more, separates into two major components.

(S_4) corresponding to the soluble proteins and containing the SRNA

(P_4) containing the bulk of the cellular free ribosomes.

Measurement of amino acid incorporation

(a) *Incorporation into protein and nucleic acid fractions.* Cell fractions, incubated

* Medium devised by NISMAN as a substitute for Pennassay medium.

** Produced by the Minnesota Mining and Manufacturing Co., St. Paul, Minn.

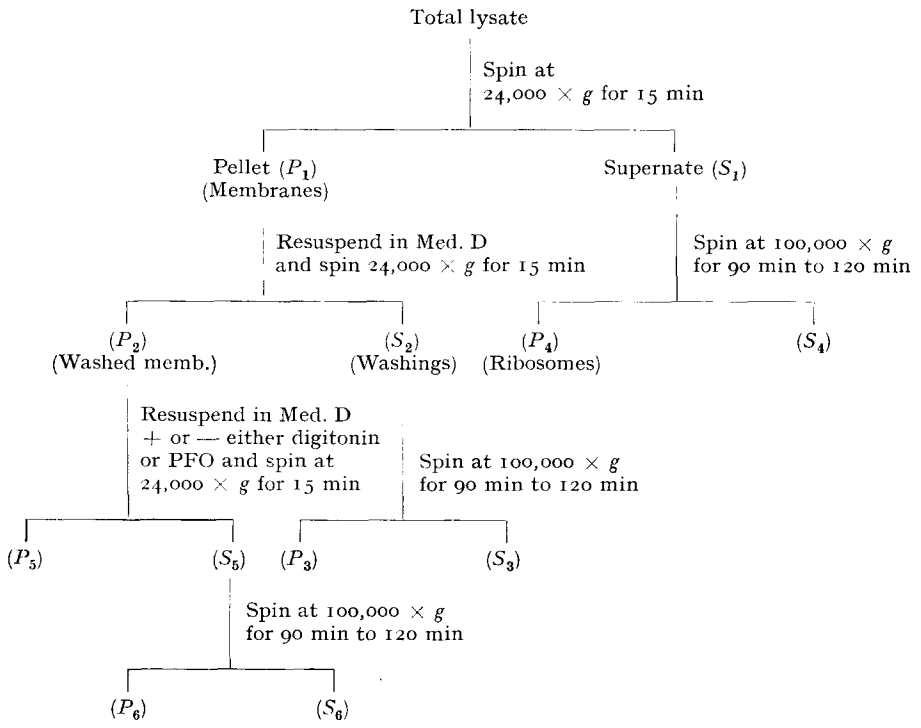

Fig. 1. Centrifugal fractionation of total lysate.

in the presence of radioactive amino acids, were precipitated with 5% TCA in the cold and were allowed to flocculate for about one hour. Approximately 1 mg of protein carrier was added and each precipitate was washed 5 times with 5% TCA in the cold. Pellets were dissolved in 0.7 *N* NH_4OH and aliquots were dried on aluminium planchets for counting. These are referred to as "total counts".

(b) *Incorporation into protein.* The amino acid–RNA bond is hot acid-[14,17], alkali-[13,14] and hydroxylamine (NH_2OH)-[14,17] labile. In experiments with *E. coli*, NISMAN[20] found that the hot acid (5% TCA) stable count is equivalent to that obtained by treatment with NH_2OH (*M*) at pH 7.0.

To insure complete solubilization of the amino acids bound to RNA, the ammonium hydroxide solubilized pellets were neutralized, and the NH_2OH concentration was adjusted to 1 *M*. Samples were left at room temperature for a minimum of one hour, precipitated, washed with 5% TCA in the cold, and then dissolved in 0.7 *N* NH_4OH. Aliquots were plated and counted. These are referred to as "NH_2OH-stable" counts and presumably represent amino acid incorporated into protein.

All samples were counted at infinite thinness in a Nuclear gas flow counter.

Measurement of [^{32}P]PP incorporation into ATP

[^{32}P]PP exchange with ATP was measured according to the technique of BERG[21] as modified by NISMAN AND HIRSCH[16].

Analytical procedures

For the determination of protein and nucleic acid, samples were precipitated and washed with 5% TCA in the cold. Pellets were dissolved in 0.7 *N* NH_4OH and assayed for protein according to LOWRY *et al.*[22].

Duplicate pellets were hydrolysed with 5% TCA at 95° for 20 minutes. The supernatants were analysed for RNA according to CHARGAFF *et al.*[23] and MEJBAUM[38], DNA according to BURTON[24].

Preparation of soluble RNA

Soluble RNA was prepared from the 100,000 × *g* supernatants (S_4), by the addition of an equal volume of water-saturated phenol at 4°, followed by precipitation of the RNA from the aqueous phase with 2 volumes of ethanol[20], and the removal of the traces of phenol.

Compounds used

The non-radioactive L-amino acids were obtained from the California Foundation for Biochemical Research. The equilibrated mixture, containing the L-amino acids in ratios corresponding to their occurrence in *E. coli* proteins, was a modification of the one previously described[8]. ATP and the four ribonucleoside diphosphates were products of the Sigma Chemical Company. Lysozyme twice crystallized, product of Armour & Co. RNA-ase crystalline pancreatic, product of Worthington & Co. Digitonine (crystalline) Merck, Darmstadt. DL-[^{35}S]methionine, Radiochemical Centre, Amersham, England. L-[^{14}C]alanine uniformyl labelled, from the Radiocarbon Laboratory of the Pasteur Institute (Drs. Aubert and Milhaud) Paris. [^{32}P]PP prepared by alkaline fusion at 400° for 60 min and separated on Dowex 1-Cl according to BERG[21].

RESULTS

Distribution of protein and nucleic acid

Cell fractions obtained from osmotically shocked protoplasts were analysed for RNA, DNA, and protein. The results (Table I) show that the fraction including the washed membranes (P_2) of *B. megaterium* contains an average of 17% of the cell protein, 12% of the cell RNA, and 37% of the cell DNA. This fraction has the highest DNA/protein ratio (0.12) and an RNA/protein ratio (0.11) lower than that of either

TABLE I

DISTRIBUTION OF PROTEIN AND NUCLEIC ACID IN ISOLATED CELL FRACTIONS

The results represent an average of at least two experiments and are expressed as % of total lysate (*TL*), and ratio of RNA and DNA to protein.

Fraction	% *Protein of TL*	% *RNA of TL*	% *DNA of TL*	$\frac{RNA}{Protein}$	$\frac{DNA}{Protein}$
TL	100	100	100	0.15	0.051
S_1	74.0	75.8	58.6	0.15	0.042
P_2 (washed membranes)	17.2	12.4	37.4	0.11	0.123
S_2 (washings)	1.6	2.9	4.5	0.24	0.102
P_4 (ribosomes)	14.5	54.4	13.8	0.59	0.048
S_4	61.9	12.1	14.3	0.031	0.013

the total lysate or S_1. The DNA content of (P_2) was variable, with DNA/protein ratios ranging from 0.09 to 0.17 obtained in three different experiments. The RNA/protein ratios varied from 0.071 to 0.12. In all cases, recoveries in the (P_2), S_1 and S_2 fractions were greater than 90%.

Essentially all of the protein and 88% of the RNA of the S_1 fraction is recovered in the fraction containing the ribosomes (P_4) and S_4 fractions. Since the recovery of DNA is only 48%, the results suggest the presence of a DNAase in the S_1 fraction, which becomes activated when the ribosomes are removed by centrifugation. (P_4) contains approximately 72% of the RNA of the S_1 fraction and has an RNA/protein ratio (0.59) greater that that of any other cell component.

As the result of one washing with Medium D, there is a preferential removal of RNA from the (P_2) fraction. The washings (S_2) contain 9–10% of the protein and DNA present in the unwashed membranes (P_1) and 20% of the RNA.

Effect of digitonin on protoplasts and membranes

As is shown in Fig. 2, the addition of 1.0 mg of digitonin to a protoplast suspension in hypertonic sucrose results in a rapid decrease in optical density (curve 3). Observations with the phase contrast microscope when the optical density had fallen to about 35% of its original value, revealed the presence of granules and cytoplasmic membranes, but the complete absence of protoplasts. When the optical density had fallen to 20% of its original value, only granules were observed. Thus, digitonin induces both protoplast lysis and membrane fragmentation. The speed of lysis depends upon the digitonin/protoplast ratio, as is shown by the increase in rate of

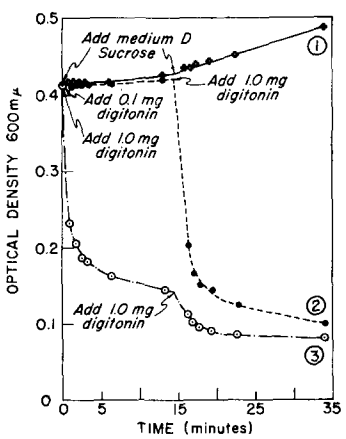

Fig. 2. Digitonin-induced lysis of protoplasts in hypertonic sucrose. Each cuvette contained 3.0 ml of a washed protoplast suspension in medium D sucrose (0.25 *M* sucrose). Digitonin was dissolved in medium D sucrose and all additions to cuvettes were made in 0.01 ml quantities. At zero time medium D sucrose was added to cuvette 1, 0.1 mg digitonin to cuvette 2, and 1.0 mg digitonin to cuvette 3. These additions were repeated after 14½ min, except that 1.0 mg digitonin was added to cuvette 2. Temp. = 24°. The optical density was read at 600 mμ against a water blank.

decline when an additional 1.0 mg of digitonin is added (curve 3). The presence of 0.1 mg of digitonin (curve 2) induces little protoplast lysis. The control protoplast suspension (curve 1) increases in optical density during the incubation period, owing to protoplast contraction.

The exposure of washed "membranes" (P_2) to either digitonin or perfluorooctanoate at 4° also results in fragmentation. Although neither membranes nor membrane fragments can be detected with the phase contrast microscope, some of the fragments are large enough to be sedimentable at 24,000 × *g*. As much as 30% of the P_2 protein may be recovered as large fragments.

Incorporation of labelled amino acids

Membrane fragments from protoplasts lysed with digitonin at 4° were tested for their ability to incorporate radioactive amino acids. The results (Fig. 3) show that both the total count and the NH_2OH-stable count increase over a period of 2 hours. The NH_2OH count varies from 50–60% of the total count. Both types of incorporation are stimulated by ATP, by a mixture of all four ribonucleoside diphosphates or triphosphates, and by a preparation of S-RNA from *E. coli*. During the first 90 minutes, S-RNA is not as effective as a mixture of ribonucleoside diphosphates in stimulating incorporation. This is consistent with the finding of, at most, traces of amino-acid-activating enzymes in "membranes" and suggests that this stimulation is due to nucleotides derived from S-RNA.

The major cell fractions were tested for their ability to incorporate amino acids. The results (Table II) show that of all components of a total lysate, the "membranes" are the most active in incorporating amino acids into a NH_2OH-stable form. While the activity of the S_1 fraction is only slightly lower than that of the total lysate, the ribosome (P_4) and S_4 fractions have the poorest incorporating abilities. The NH_2OH-labile counts associated with the "membrane" are lower than those associated

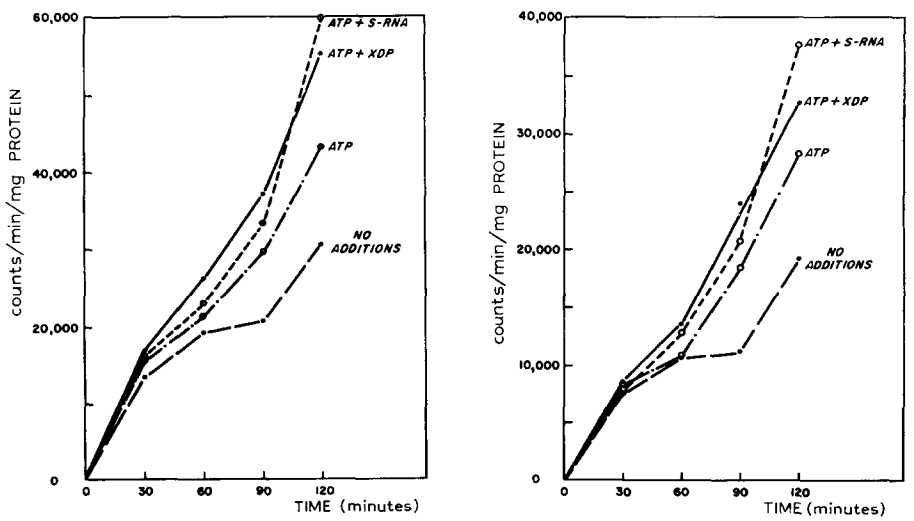

Fig. 3. Incorporation of [^{35}S]methionine and [^{14}C]alanine by membrane fragments. 3A = total incorporation; 3B = NH_2OH stable incorporation. The membrane fragments were obtained by digitonin lysis of protoplasts and were used without washing. The reaction mixtures were incubated aerobically with shaking at 30°. Each flask contained the following constituents per ml: 540 μg membrane protein, 55 μmoles Tris buffer pH 7.3, 1.1 μmoles $MgSO_4$, 0.1 μmoles $MnCl_2$, 260 μg equilibrated amino acid mixture, 66 mμmoles L-[^{35}S]methionine of 7,500 counts/min/mμmole, and 10 mμmoles L-[^{14}C]alanine of 3,800 counts/min/mμmole. Additions per ml: 0.55 μmoles ATP, 44.4 μg XDP mixture containing 11.1 μg of each of the four ribonucleoside diphosphates, and 222 μg S-RNA from *E. coli*.

TABLE II

LOCALIZATION OF THE AMINO-ACID-INCORPORATING SYSTEM

The cell fractions were obtained from osmotically lysed protoplasts. The membrane fraction was washed once. The reaction mixtures were incubated aerobically at 30°. Each mixture contained the following constituents per ml: 35 μmoles Tris buffer pH 7.3, 1.0 μmole $MgSO_4$, 1.0 μmole $MnCl_2$, 0.52 μmoles ATP, 41.6 μg XDP mixture containing 10.4 μg of each of the four ribonucleoside diphosphates, 122 μg equilibrated amino acid mixture, 220 mμmoles L-[^{35}S]methionine of 750 counts/min/mμmole, and the following amounts of protein: 207 μg *TL*, 202 μg S_1, 144 μg P_2, 350 μg P_4, and 680 μg S_4. The results are expressed as the change in specific activity over a period of 80 min.

Fraction	*CPM/mg Protein*		
	Total counts	*NH_2OH-stable count*	*Counts liberated by NH_2OH*
TL (total lysate)	5710	1867	3843
S_1	5500	1703	3797
P_2 (membranes)	3720	2780	940
P_4 (ribosomes)	2900	1010	1890
S_4	2656	716	1940

with either the total lysate or S_1 fractions. This is consistent with the lower RNA content of "membranes".

(P_2) fraction obtained by osmotic lysis of protoplasts was exposed to either digitonin or PFO at 4°. The resulting large fragments were recovered by centrifugation at 24,000 × *g* and the incorporating ability of these fragments was compared with that of a twice-washed "membrane" preparation. The results (Table III) show that both types of fragments incorporate amino acids more rapidly than the "membrane" control (P_5A). In all cases, S-RNA of *B. megaterium* stimulates both the total incorporation and the NH_2OH stable incorporation, while the equilibrated amino acid mixture has an inhibitory effect. In contrast to the first experiment on incorporation (Fig. 3), ATP has only a slight effect on the NH_2OH stable count, and often an inhibitory effect on the total count.

The results of a typical experiment on the effect of RNAase on the incorporating ability of (P_2) are presented in Table IV. RNA-ase, added at zero time, has a slight inhibitory effect on the total incorporation and has essentially no effect on the NH_2OH stable count. Soluble RNA stimulates both types of incorporation, especially in the presence of RNA-ase. These findings suggest that S-RNA stimulates by virtue of its ability to act as a substrate for RNA-ase and thereby furnishes nucleotides.

Amino acid activation

Cell fractions, obtained by osmotic lysis of protoplasts, were tested for their ability to catalyse an amino-acid-dependent PP exchange with ATP. The results of 2 experiments are summarized in Table V. To facilitate the assay of many cell fractions, mixtures of amino acids were used. Although the fractions were not dialysed to remove free amino acids, stimulations in PP exchange were observed upon the addition of amino acid mixtures. This was true even of fractions expected to contain the highest endogenous amino acid levels (*TL*, S_1).

It is clear that most of the amino-acid-stimulated exchange activity of the total lysate can be accounted for by the S_1 fraction. In fact, in the presence of the

TABLE III

EFFECT OF DIGITONIN AND PERFLUORO-OCTANOATE TREATMENT ON INCORPORATING ABILITY OF MEMBRANES

Cell membranes, obtained from osmotically lysed protoplasts, were washed once. The membranes (P_2) were then incubated with either medium D alone, or with medium D containing digitonin at a final concentration of 3 mg/ml, or PFO at a final concentration of 9 mg/ml. After 20 min at 4°, the preparations were centrifuged at 24,000 × *g* for 15 min, and the resulting pellets (P_5) tested for ability to incorporate amino acids. The reaction mixtures were incubated aerobically with shaking at 30°. Each flask contained the following constituents per ml: 65 μmoles Tris buffer pH 7.3, 1.5 μmoles $MgSO_4$, 1.5 μmoles $MnCl_2$, and 220 mμmoles L-[^{35}S]methionine of 750 counts/min/mμmole. Additions per ml: 0.62 μmoles ATP, 535 μg equilibrated amino acid mixture (without methionine), 74 μg S-RNA from *B. megaterium*, and the following amounts of protein: 231 μg P_5A (Medium D-treated), 234 μg P_5B (digitonin-treated), and 204 μg P_5C (PFO-treated).

Additions	*Treatment*	*Fraction*	*Time (min)*	*Counts/min/mg Protein*	
				Total counts	*NH_2OH-stable count*
—	Medium D	P_5A	40	4500	2090
			80	7000	2500
ATP		P_5A	40	3480	1870
			80	5050	3030
ATP, S-RNA		P_5A	40	4470	2840
			80	5910	2620
ATP, AA		P_5A	40	2960	1660
			80	3540	2010
—	Digitonin	P_5B	40	6200	2770
			80	6770	3810
ATP		P_5B	40	5640	2770
			80	7340	3300
ATP, S-RNA		P_5B	40	7110	3680
			80	9170	4420
ATP, AA		P_5B	40	4310	2830
			80	4440	2830
—	PFO	P_5C	40	6440	2450
			80	6170	3530
ATP		P_5C	40	4940	2550
			80	6450	3730
ATP, S-RNA		P_5C	40	6380	3870
			80	7160	5100
ATP, AA		P_5C	40	4710	2160
			80	5100	2800

most active amino acid mixtures, the specific activity of the S_1 fraction is greater than that of the total lysate. The washed membranes (P_2) contained small amounts of activity in Expt. 4 and were completely inactive in Expt. 5. In the latter experiment, the addition of amino acid mixtures inhibited the endogenous PP exchange activity

TABLE IV

EFFECT OF RNAASE ON INCORPORATING ABILITY OF MEMBRANES

Cell membranes, obtained from osmotically lysed protoplasts, were washed once. The reaction mixtures were incubated aerobically with shaking at 30°. Each flask contained the following constituents per ml: 37 μmoles Tris buffer pH 7.3, 1.0 μmole $MgSO_4$, 1.0 μmole $MnCl_2$, 334 μg equilibrated amino acid mixture, 117 mμmoles L-[^{35}S]methionine of 2,010 counts/min/mμmole, and 161 μg membrane protein. Additions per ml: 143 μg RNAase, and 60 μg S-RNA (mixtures of equal parts from *E. coli* and *B. megaterium*).

Additions	*Time (min)*	*Counts/min/mg Protein*	
		Total counts	*NH_2OH-stable count*
—	40	2735	1380
	80	2670	1520
RNAase	40	2220	1370
	80	2550	1560
S-RNA	40	2920	1560
	80	3600	1930
RNAase, S-RNA	40	2980	920
	80	7770	3260

of the P_2 fraction. Results similar to those of Expt. 5 were obtained in a third experiment. Activating enzymes are readily removed from "membranes" by washing, as is shown by the finding of low levels of activity in the S_2 fraction and none in the P_2 fraction (Expt. 5). This suggests that the "membranes" of Expt. 4 were improperly washed and were, therefore, contaminated with the S_1 fraction.

About 80% of the activity of the S_1 fraction is recovered in the ribosome (P_4) and S_4 fractions, with S_4 somewhat more active than P_4. The low levels of amino-acid-stimulated activity associated with P_2 in Expt. 4 are lost after fragmentation of the membranes with digitonin. The large fragments (P_5B) have a slightly higher level of endogenous PP exchange than the P_2 fraction. However, values below the endogenous level are obtained when amino acid mixtures are added to P_5B.

DISCUSSION

The fraction including the membrane (P_2) of *B. megaterium* strain M contains an average of 12% of the cell RNA and 37% of the cell DNA. BUTLER *et al.*[6] reported that the membranes of *B. megaterium* strain KM contain 13% of the cell RNA and 54% of the DNA (data calculated from their paper). NISMAN AND FUKUHARA[37] reported that the fraction containing the membrane fragments in *E. coli* contains 80–85% of the total cell DNA. However, there have been conflicting reports as to the presence of nucleic acid in bacterial membranes. VENNES AND GERHARDT[25] found RNA, but no DNA, in the membranes of strain KM, while GILBY *et al.*[27] could find no more than traces of nucleic acid in the membranes of *Micrococcus lysodeikticus*. WEIBULL *et al.*[26] originally reported that nucleic acids were essentially absent from the membranes of strain M and subsequently reported that membranes of this strain contain no more than 5% of the cell RNA[2]. In addition, there have been reports

TABLE V

LOCALIZATION OF THE SYSTEM CATALYZING THE AMINO-ACID-DEPENDENT EXCHANGE OF PP WITH ATP

The cell fractions were obtained from osmotically lysed protoplasts. Expt. 4. An aliquot of washed membranes (P_2) was incubated with digitonin (final concentration of 4.5 mg/ml) at 4° for 15 min and then centrifuged at 24,000 × *g* for 15 min. The supernatant (S_5B) was then centrifuged at 100,000 × *g* for 90 min. The pellets (P_3B and P_6B) were resuspended in medium D, while the protein of S_6B was precipitated with 1 vol. of ethanol and then suspended in medium D. Each reaction mixture contained the following constituents per ml: 100 μg protein of each fraction tested, 100 μmoles Tris buffer pH 8.0, 5.0 μmoles $MgSO_4$, 10.0 μmoles KF, 2.0 μmoles ATP, and 1.75 μmoles sodium pyrophosphate of 27,500 counts/min/μmole. Mixtures of L-amino acids were added to give a final concentration of 2.0 μmoles of each amino acid per ml. Reaction mixtures were incubated for 15 min at 30° and the reaction was stopped by adding cold TCA. Expt. 5. Reaction mixtures contained the same constituents as those used in Expt. 4, except that the sodium pyrophosphate concentration was increased to 2.0 μmoles/ml (58,900 counts/min/μmole). Mixtures were incubated for 15 min at 30° and the reaction was stopped by adding cold TCA.

mM moles [^{32}P]PP incorporated into ATP per mg protein

Fraction	*Expt. 4*						*Expt. 5*					
	Amino acids added						*Amino acids added*					
	none	*ala arg glu glu–NH_2 gly*	*thr asp asp NH_2 ser lys*	*cys–SH meth*	*val leu ileu*	*pro phe try tyr his*	*none*	*ala arg glu glu–NH_2 gly*	*thr asp asp–NH_2 ser lys*	*cys SH meth*	*val leu ileu*	*pro phe try tyr his*
TL	364	378	320	524	712	528	237	356	485	700	1470	509
-Endog		14	—	160	348	164		119	248	463	1233	272
S_1	375	295	346	618	1465	488	142	314	440	875	1720	505
-Endog		—	—	243	1090	113		172	298	733	1578	363
P_2	269	306	287	298	324	331	95	20	10	20	65	27
-Endog		37	18	29	55	62		—	—	—	—	—
S_2							41	34	44	88	163	54
-Endog								—	3	47	122	53
P_4	135	218	156	269	222	182						
-Endog		83	21	134	87	47						
S_4	204	207	193	389	582	197						
-Endog		3	—	185	378	—						
P_3B	298	84	76	171	167	164						
-Endog		—	—	—	—	—						
P_6B	95	58	134	98	69	91						
-Endog		—	39	3	—	—						
S_6B	182	87	149	142	142	80						
-Endog		—	—	—	—	—						

of the association of nuclear bodies with the membrane fraction of strain KM[28,29]. These bodies contain RNA, DNA, and protein in a ratio of 1:1:3. At present, it is difficult to explain the contradictory findings as to the presence of nucleic acid in membranes or to determine to what extent the finding of DNA in "membranes" indicates contamination with nuclear bodies.

The endogenous PP exchange activity of the membrane fraction is lower than that of either the total lysate or the S_1 fraction. This endogenous activity could be due to the presence of amino acids, fatty acids[14,30], inorganic acids[31,32], or to the residual PP exchange activity found in dialysed extracts[33]. However, the addition of amino acid mixtures to the total lysate and S_1 fraction does result in an increased rate of PP exchange. These fractions would be expected to contain the highest endogenous amino acid levels. No such stimulation was observed in two experiments with (P_2) and only a slight stimulation was observed in a third experiment. This low level of activity was lost after fragmentation of the membranes with digitonin. Therefore, "membranes" capable of incorporating amino acids into a NH_2OH-stable form do not necessarily contain amino-acid-activating enzymes, as measured by PP exchange with ATP. They may contain small amounts of these enzymes if they are improperly washed. Experiments are now in progress to determine whether or not the activating enzymes have any effect on the amino acid incorporation by "membranes".

Although the S_1 fraction has appreciable amino-acid-incorporating ability, it is clear that the fraction containing the membranes is the most active component of the total lysate. The activity of the "membranes" is stimulated by S-RNA from either *B. megaterium* or *E. coli*, a mixture of all four ribonucleoside diphosphates, or by fragmentation with either digitonin or PFO. The stimulation in both the total and NH_2OH stable incorporation as the result of digitonin fragmentation, suggests that amino acid activation plays no role in either type of incorporation. "Membranes" obtained by osmotic lysis of protoplasts show a variable response to ATP. When "washed membranes" (P_2) are incubated with ATP, there is a 7% increase in the NH_2OH stable count and a 25% decrease in the total count (Table III). In an experiment with membranes from digitonin lysed protoplasts (Fig. 3), ATP induced a 50–60% stimulation in both types of incorporation. However, the membranes of the latter experiment were not washed.

In experiments with "membranes" the NH_2OH-stable count is usually 50–60% of the total count. The amino acids solubilized by NH_2OH treatment at pH 7.0 could be bound to ribonucleoprotein or to something analogous to the lipid-soluble amino acid complex of hen oviduct[34]. Since the presence of RNA-ase causes only a small decrease (15%) in the total count (Table IV), little of the amino acid can be linked to S-RNA. Also, this linkage would presumably occur via a mechanism not involving amino acid activation, as measured by PP exchange with ATP, or by subsequent formation of S-RNA-AA.

The incorporation of [^{35}S]methionine by "membranes" is depressed by the presence of a complete amino acid mixture. There is a decrease in both the total, and the NH_2OH-stable incorporation. The incorporation by *E. coli* membranes[7,8,16] is stimulated by the presence of all the other amino acids, while the complete amino acid mixture is without effect on the incorporation by rat liver supernatant[15]. Findings of inhibition are not too surprising, in view of recent evidence for the variable amino

acid composition of bacterial proteins, in the presence of either naturally occurring amino acids or analogues[35].

The amino-acid-incorporating activity of the *B. megaterium* membrane is insensitive to RNA-ase, while the activity of phenol-treated *E. coli* membrane fragments is stimulated by RNA-ase[17]. Both systems, like *A. faecalis* membrane fragments[9] and PFO-disrupted rat liver microsomes[36], incorporate amino acids under conditions in which amino-acid-activating enzymes cannot be demonstrated.

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