Cellular Components of *Bacillus megaterium* and their Role in Protein Biosynthesis

By G. N. GODSON, G. D. HUNTER* AND J. A. V. BUTLER

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital,
Fulham Road, London, S.W. 3

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Previous reports from this Laboratory (Butler, Crathorn & Hunter, 1958; Hunter, Brookes, Crathorn & Butler, 1959) have described the preparation of a membrane complex ('cytoplasmic membrane fraction') from protoplasts of *Bacillus megaterium*. When prepared under defined conditions, the membrane complex contained the complete apparatus necessary for the incorporation of labelled amino acids into polypeptide bonds. Intermediate reactions in the process were also studied and it was shown that the membrane complex contained enzymes, capable of activating amino acids, and also RNA that binds amino acids into bonds labile to the action of dilute alkali and ribonuclease. In the present work, the nature of the membrane complex has been examined in greater detail, and it has been possible to separate several components by mild physical methods. These components, which include ribonucleoprotein particles and a phospholipoprotein fraction, have been analysed by both physical and chemical methods, and an attempt has been made to assess their individual contributions to the protein-synthetic mechanism.

METHODS

Organism. The strain of *B. megaterium* used (originally KM) was cultured as described by Butler *et al.* (1958). For all the experiments described here the organism was grown at 30° in a glucose-salts medium (C medium; McQuillen, 1955).

Preparation of the membrane complex and cytoplasmic fraction. Protoplasts were prepared by gentle shaking at 30° in the presence of 0.02% lysozyme and in a medium of the following composition (% w/v), finally brought to pH 7.0 with NaOH: glucose, 1; NH₄Cl, 0.2; Na₂HPO₄, 0.6; KH₂PO₄, 7.15; NaCl, 0.3; Na₂SO₄·10H₂O, 0.025; MgCl₂·6H₂O, 0.222 (this will be termed below as 0.5M-phosphate-salts medium). Cells from early logarithmic-phase cultures were usually completely converted into protoplasts within 30 min. When microscopic examination failed to reveal the presence of a single whole cell in a sample, the protoplasts were harvested and then osmotically lysed in one of two ways.

In the procedure designated 'conc. lysis' the protoplast pellet (1–2 g.) was resuspended in 1 vol. (80 mg./ml.) of

0.5M-phosphate-salts medium, diluted with 1.5 vol. of water and vigorously shaken by hand until no whole protoplasts could be seen under the microscope. When the weight of protoplasts was smaller (0.2–0.5 g.), the pellet was immediately suspended in the diluted medium and shaken as before. The resulting suspension was centrifuged at 105 000g for 10 min. to sediment the membrane complex, the supernatant being termed the cytoplasmic fraction.

In the procedure designated 'dil. lysis', the protoplasts were lysed as above, but in this case at least 15 vol. of water was used. Lysis under these conditions is very rapid and the membrane complex and cytoplasmic fractions were collected as before. In experiments where it is undesirable to use phosphate media (e.g. experiments on pyrophosphate exchange), tris (brought to pH 7.2 with HCl) was used at final concentration of 0.6M for 'conc. lysis' and 0.01M for 'dil. lysis'.

Fractionation of the membrane complex. The membrane complex was disintegrated in buffered salt media by a variety of methods. The normal procedure was to treat the membrane complex (approx. 50 mg./ml.) with ultrasonic vibrations (from an M.S.E.-Mullard machine) for 3 min. The operation was performed in a plastic tube (12 ml.) cooled in an ice bath. In early experiments, a Mickle glass-bead tissue disintegrator was used, but it was subsequently found that the same result could be obtained simply by shaking the membrane complex vigorously by hand until it had been visibly dispersed. Provided the membrane complex is completely dispersed with no large lumps visible, the method and the volume of solution are unimportant. Ultrasonic disintegration was preferred in these experiments as the volume can be kept small and the operation can be carried out rapidly. The treatment was usually carried out in 0.03M-phosphate-salts medium or 0.01M-tris and the concentration of Mg brought to 0.01M.

The resulting suspension was centrifuged at 50 000g for 25 min. The supernatant fraction was removed, the tube cut, and the two layers of the sediment were separated with a spatula. The lower layer mucoprotein fraction was so hard packed that the upper layer (phospholipoprotein fraction) could be removed without contamination. If a clean uncontaminated sample of the mucoprotein fraction was required, it was resuspended and resedimented, and the fine film of the top layer removed.

The supernatant fraction removed from the two sedimented layers contained ribonucleoprotein particles (ribosomes, 30s and 45s) as well as free protein and RNA (approx. 5s). These components were separated in a discontinuous sucrose-density gradient. The sucrose solutions also contained 0.01M-MgCl₂ and 0.03M-KH₂PO₄ adjusted to pH 7.0 with NaOH. The density gradient was constructed by

* Present address: A.R.C. Field Station, Compton, Nr. Newbury, Berks.

layering 8 ml. each of solutions containing 1.25M-, 0.75M- and 0.5M-sucrose in a centrifuge tube about 1 hr. before use. All the operations were performed at 2–5°. The solution containing ribosomes (3–4 ml.) was layered on the top, and the gradient spun at 20 000 rev./min. in a Spinco SW. 25 head for more than 6 hr. The ribosomes sediment to a visible band about two-thirds of the way down the tube while the free protein and RNA barely pass the boundary into the 0.5M-sucrose. The bottom of the tube was pierced with a hypodermic needle and the emergent drops were collected in a series of fractions. A sample of each fraction was diluted. The extinction coefficients at 260 m μ indicated the distribution of three distinct separated components, namely 30s, 45s and 5s material. Each fraction was normally precipitated directly with cold 30% trichloroacetic acid to a final concentration of 5%, and the precipitate recovered by centrifuging. If desired the ribosomes can be sedimented out of the sucrose media by centrifuging at 105 000g for 4 hr.

Experiments with the isolated membrane complex. When the uptake of [14 C]amino acids by the various fractions of the membrane complex was being studied, the membrane pellet was thoroughly shredded into small pieces with a spatula and then resuspended in 50 ml. (2 mg./ml.) of 0.5M-phosphate-salts medium and equilibrated by shaking at 30° for 30 min. To terminate the incubation 0.2 mg./ml. of cold chloramphenicol was added and the incubation flask plunged immediately into an ice bath. Such treatment inhibits the incorporation of [14 C]amino acids within 2–3 sec. The fragments of the membrane complex are then spun out of the incubation mixture by centrifuging in the cold at 105 000g for 5 min. The labelled membrane complex is then separated into its components as indicated above. The presence of chloramphenicol at this stage is unimportant and does not affect the distribution of label among the fractions.

Experiments with protoplasts. In later experiments, protoplasts were employed because they normally incorporate [14 C]amino acids faster than the isolated membrane complex and the conditions of protein synthesis are nearer to those of a whole cell. The protoplasts were separated in 0.5M-phosphate-salts medium (about 50 mg./ml.), equilibrated by shaking at 30° for 1 hr. and incubated with [14 C]amino acids, and the labelling was terminated as for the membrane complex. The protoplasts were harvested in the cold and lysed in concentrated buffer in the presence of chloramphenicol (0.2 mg./ml.), and the membrane complex obtained was fractionated as above. The label in the cytoplasmic fraction was also recorded in these experiments. The protoplasts are quite stable in 0.5M-phosphate-salts medium and can be fairly vigorously shaken.

Safeguards against contamination with whole cells. *B. megaterium* is so large (5 μ) that whole cells can easily be seen under the high power of a phase-contrast microscope ($\times 1000$). Preparations of protoplasts were never used if a single whole cell could be found in a sample of the lysozyme digest. Similarly, preparations of the membrane complex were examined for whole cells. A further indication that no whole cells were present was the very small amounts of radioactivity incorporated into the mucoprotein fraction. Whole cells would have remained unbroken by the mild fractionation procedure and would have sedimented with the mucoprotein fraction.

Experiments on amino acid-activating enzymes. To test for

amino acid-activating enzymes the cell fractions were prepared in phosphate-salts or tris media, as indicated in the text. The final incubation mixture contained 2 mM-KF, 2 mM-MgCl₂, 2 mM-ATP, a mixture of amino acids giving a final concentration of 4 mM, an enzyme preparation (about 80 μ g. of protein/ml.) in a final volume of 2 ml. buffered to pH 7.2 with tris or phosphate-salts medium. Incubations were carried out at 37° for 30 min. in the presence of 0.25 μ C of sodium [32 P]pyrophosphate, the reaction was terminated with 2 ml. of 10% trichloroacetic acid, and the ATP isolated by absorption on charcoal as described by Crane & Lipmann (1953). The washed charcoal was finally boiled for 10 min. with N-HCl and the radioactivity of the hydrolysate determined.

Assays of radioactivity. The protein samples were prepared for radioactive assay by the methods previously described (Butler *et al.* 1958; Crathorn & Hunter, 1957). Assay of solid samples was carried out by end-window counting as described by Butler *et al.* (1958).

32 P samples were counted in 10 ml. samples in an annular liquid counter Type M6M (20th Century Electronics Ltd., New Addington, Surrey).

Chemical analyses

Estimation of ribonucleic acid. RNA was estimated by the orcinol method (Ceriotti, 1955). The results were checked on another sample by measurement of the extinction at 260 m μ in a Unicam spectrophotometer (SP. 600) after extraction of the RNA by the Schmidt-Thannhauser procedure, and with an extinction coefficient of 32.4 mg./ml./cm. (Scott, Fraccastoro & Taft, 1956).

Estimation of protein. In all fractions except the bottom sediment, which contains a large amount of carbohydrate, the weight of the material insoluble in hot trichloroacetic acid after extraction of the lipids was taken as the weight of protein. This was checked by determining the protein according to Lowry, Rosebrough, Farr & Randall (1951), insoluble protein samples being dissolved in N-NaOH. The protein contents of the particulate fractions were checked by determining the N content by a micro-Kjeldahl method and multiplying by 6.5. Standard curves, which were closely similar, were prepared from lysozyme, ribonuclease or serum albumin, all dissolved in N-NaOH. All the final mixtures contained the same amount of alkali.

Estimation of lipid. The lipid was determined by the loss in weight of a sample after extraction by boiling for 30 min. in chloroform-ethanol (1:1, v/v). The extract was then evaporated to dryness and the residue weighed to check the first estimate. The inorganic phosphorus present in the extract was determined after destruction of all organic material with 60% (w/w) HClO₄ (A.R.) by the method of Fiske & Subbarow (1925). By using 3.5% as the phosphorus content of phospholipid (Weibull & Bergström, 1958) the phospholipid can be calculated. Some protein is solubilized by the chloroform-ethanol solvent so that the lipid content is slightly high by the weight difference method, but, as *B. megaterium* contains some neutral lipid (Hunter & Good-sall, 1961), this method may be more accurate than that of Weibull & Bergström (1958).

Electron microscopy. Samples were prepared and fixed by the methods described by Mercer (1959).

Sedimentation in the ultracentrifuge. The sedimentation velocity experiments were performed in a Spinco model E

ultracentrifuge at 35 500 rev./min., the observed sedimentation coefficients being corrected to water at 20° in the normal way.

Materials. Generally labelled L- $[^{14}\text{C}]$ amino acids (5–9 $\mu\text{C}/\mu\text{mole}$) were supplied by The Radiochemical Centre, Amersham, Bucks., and were used as supplied without any dilution with $[^{14}\text{C}]$ amino acids.

Sodium $[^{32}\text{P}]$ pyrophosphate was also obtained from The Radiochemical Centre.

RESULTS

Components of the membrane complex. Protoplasts of *B. megaterium* can be lysed in two ways: either by shaking in a dilute buffer (0.03M-phosphate-salts or 0.01M-tris) when the lysis is instantaneous or by diluting a concentrated buffer medium just sufficiently to effect osmotic rupture (0.3M-phosphate-salts or 0.6M-tris). The two processes are termed below 'conc. lysis' and 'dil.

lysis'. It has been found that the slow-speed sediment (membrane complex) obtained after conc. lysis of the protoplasts is capable of incorporating $[^{14}\text{C}]$ amino acids into protein peptide bond, but the membrane complex obtained by dil. lysis is unable to do this. In each case about 50% of the total cellular RNA is deposited with the membrane complex, and, if this complex is washed with a dilute buffer (0.03M-phosphate-salts or 0.01M-tris, each containing 0.01M-MgCl₂ and adjusted to pH 7.2 with NaOH or HCl), 30s and 45s ribosomes are released together with amino acid-activating enzymes, free protein and RNA (Figs. 1 and 2; Table 2). The RNA analyses given in Table 1 show that approximately 57% of the RNA present in the protoplasts is ribosomal and after dil. lysis about 67% of these ribosomes are sedimented with the membrane complex. Approximately 70% of the RNA bound to the membrane complex (both dil.

(a)

(b)

Fig. 1. Sedimentation diagrams of fractions obtained when protoplasts are lysed in 0.03M-phosphate-salts medium. (a) Cytoplasmic fraction, photographed at 12 min. after attainment of full speed. (b) Material released from membrane complex when it is washed in 0.01M-tris + 0.01M-MgCl₂, pH 7.2, photographed at 28 min. after attainment of full speed. For full experimental details, see text.

(a)

(b)

Fig. 2. Sedimentation diagrams of fractions obtained when protoplasts are lysed in 0.3M-phosphate-salts medium. (a) Cytoplasmic fraction. (b) Material released from membrane complex when it is washed in 0.01M-tris + 0.01M-MgCl₂, pH 7.2. Both photographs taken at 16 min. after attainment of full speed. For full experimental details, see text.

Table 1. *Distribution of ribosomal and non-particulate ribonucleic acid in fractions of lysed protoplasts*

The conditions of lysis and methods for chemical analysis were as described in the text.

		Conc. lysis (0.6 M-tris, 0.01 M-MgCl ₂)		Dil. lysis (0.01 M-tris, 0.01 M-MgCl ₂)	
		RNA (mg.)	Percentage of total	RNA (mg.)	Percentage of total
Cytoplasmic fraction	RNA in ribosomal form	0.7	1.7	9.4	19.6
	RNA in non-particulate form	19.4	47.2	14.2	29.8
Membrane complex	RNA in ribosomal form	16.8	40.6	18.2	38.1
	RNA in non-particulate form	4.3	10.5	6.1	12.5

Table 2. *Distribution of amino acid-activation enzymes among fractions derived from protoplasts of Bacillus megaterium*

The various fractions were prepared and assayed as described in the Methods section of the paper. The incubation mixtures were buffered with phosphate-salts medium (pH 7.2; final concn. 0.03 M).

Treatment	Fraction	Incorporation of added [³² P]pyrophosphate into ATP (%/0.5 mg. of protein)
Protoplasts lysed in 0.3 M-phosphate-salts medium	Cytoplasm	3.1
	5s-fraction of membrane complex	20
	Phospholipoprotein fraction of membrane complex	0.06
Protoplasts lysed in 0.03 M-phosphate-salts medium	Cytoplasm	15
	5s-fraction of membrane complex	29
	Phospholipoprotein fraction of membrane complex	0.04

Table 3. *Effect of ionic strength upon the activity of amino acid-activating enzymes*

The enzyme solution was prepared from a membrane complex after lysis of protoplasts in 0.3 M-phosphate-salts and the activity assayed as described in the Methods section.

	Incorporation of added [³² P]pyrophosphate into ATP (%/0.5 mg. of protein)
Enzyme solution made up to volume in 0.01 M-tris	15
Enzyme solution made up to volume in 0.3 M-tris	5
Enzyme solution made up to volume in 0.03 M-phosphate-salts	27
Enzyme solution made up to volume in 0.3 M-phosphate-salts	9

and conc. lysis) can be washed off as ribosomes, 25% as free RNA of low sedimentation constant and approximately 6% is tightly bound to the phospholipoprotein sediment. (Percentage amounts of RNA were calculated from the data presented in Table 1.) The cytoplasmic fraction obtained after

dil. lysis contains some ribosomes and amino acid-activating enzymes but is not capable of incorporating [¹⁴C]amino acids into protein (Fig. 1; Table 2). No ribosomes and very little [³²P]pyrophosphate-exchange activity can be detected in the cytoplasmic fraction obtained by conc. lysis (Fig. 2; Table 2). When, however, a solution of ribosomes suspended in a dilute buffer is dialysed (for 1 hr.) against 0.6 M-tris + 0.01 M-MgCl₂ or 0.3 M-phosphate-salts + MgCl₂ to saturation, the 30s and 45s peaks disappear and the amount of material absorbing at 260 mμ present in the 4–12s peak increases. It is probable therefore that on conc. lysis the same number of ribosomes are released into the cytoplasmic fraction as on dil. lysis, but the ionic strength is sufficiently high to cause them to break down. The RNA analyses given on Table 1 are consistent with this supposition. The ribosomes that are bound to the membrane complex after conc. lysis must be resistant to the degradative effect of high ionic strength.

The distribution of amino acid-activating enzymes after dil. and conc. lysis is given in Table 2. Table 3 shows that a high concentration of phosphate or tris buffer inhibits [³²P]pyrophosphate-exchange activity and the enzyme system is per-

Table 4. *Relative amounts of membrane complex and cytoplasmic fraction obtained after rupturing protoplasts under conditions of conc. lysis and dil. lysis*

The conditions of lysis were as described in the text.

	Dil. lysis (0.03M-phosphate-salts medium)		Conc. lysis (0.25M-phosphate-salts medium)	
	% Dry wt. (average of 12 expts.)	Sample figures from 1 expt. (mg.)	% Dry wt. (average of 12 expts.)	Sample figures from 1 expt. (mg.)
Whole protoplasts	100	61.4	100	54.3
Membrane complex	27	15.9	33	18.5
Cytoplasmic fraction	73	45.5	67	35.8

manently damaged as the activity is only partially restored by redilution of the buffer. This explains the low [32 P]pyrophosphate-exchange activity of the membrane complex and cytoplasmic fraction after conc. lysis (Table 2).

The proportion of membrane complex and cytoplasmic fraction, expressed as percentage dry weights, when protoplasts are subjected to conc. and dil. lysis are given in Table 4. The small differences are not regarded as significant.

After the ribosomes, free protein and RNA have been washed off a membrane complex, the debris sediments in two clearly defined layers. The bottom layer is heavy, white, opaque and often begins to sediment out on standing. The top layer is brown and gelatinous and requires a fairly high centrifugal force (30 000 rev./min. for 25 min.) to sediment completely, although most of it can be obtained at much lower centrifugal speeds (10 000 rev./min. for 20 min.). The top layer lies loosely on the hard-packed bottom layer and can be clearly separated from it with a spatula. No further amounts of protein, RNA or ribosomes were released when the top and bottom layers were resuspended and thoroughly washed in the presence of ultrasonic vibrations in dilute buffer. Both remained unaltered after repeated resuspension and sedimented with their usual characteristics.

Table 5 shows the proportion of material that is found in the different components of the membrane complex prepared by conc. lysis. The figure given in Table 4 for the ribosomes is somewhat arbitrary, and can be as low as 2 % of the total material of the complex derived from cells in the stationary phase of growth, and as high as 10 % when derived from fresh cells in the logarithmic phase. The proportions of the other components of the complex varies very little from experiment to experiment, being independent of the growth state of the cells and the methods of physical disintegration employed.

Chemical composition of the membrane complex. The analysis of the protein, RNA and lipid present in the various fractions of the membrane complex (prepared by conc. lysis) is given in Table 6, where

Table 5. *Relative proportions by weight of fractions produced by treating the membrane complex (obtained under conditions of conc. lysis) with dilute buffers*

The membrane complex was prepared and fractionated as described in the text.

Fraction	% Dry wt. (average of 15 expts.)	Sample figures from 1 expt. (mg.)
Whole membrane complex	100	38.01
Phospholipoprotein fraction	26	9.85
Mucoprotein fraction	50	19.72
5s-fraction of the complex	16	6.36
Ribosomes	8	3.08

the data were obtained after disrupting the membrane complex by ultrasonic treatment. Closely similar results were obtained in other experiments when the membrane complex was disrupted in simpler ways such as prolonged mechanical shaking in a dilute buffer. The brown gelatinous top layer of the sediment is made up mainly of protein (64 %), lipid (23 %) and RNA (2 %) with a little DNA (1 % by the Ceriotti, 1952, method). More material is insoluble in hot trichloroacetic acid than can be accounted for as protein. Presumably 10–15 % of carbohydrate is also present. The bottom layer contains about 80 % of material insoluble in hot trichloroacetic acid that does not appear to be proteinaceous in nature. It is probable that the bulk of this fraction is made up of carbohydrate, although about 10–20 % of protein is also present. This carbohydrate does not represent cell-wall material, since this is completely digested by the lysozyme treatment (Brookes, Crathorn & Hunter, 1959).

About 70 % of the RNA and 23 % of the protein that can be washed off by dilute buffer from the membrane complex prepared by conc. lysis is accounted for by the ribosomal fraction. The material remaining after separating the ribosomes consists of about 7 % of RNA and 80–85 % of protein, the rest being presumably mainly carbohydrate and inorganic salts. The RNA content of the ribosomes

Table 6. *Chemical composition of components of the membrane complex (prepared by conc. lysis)*

The samples were prepared and analysed as described in the text. The results are expressed as percentages of the total dry weight of each component.

	Phospholipo- protein fraction	Mucoprotein fraction	5s-fraction of the complex	Ribosomes
Material insol. in trichloroacetic acid	74	95	90	40-50
Protein determined by the Lowry method	64	17	87	40-50
Protein determined by the Kjeldahl method	55	9	—	—
RNA by the orcinol method (Ceriotti, 1952)	1-2.5	0.5-1	—	50-65
RNA from the u.v. absorption at 260 μ m	2	1	7	50-60
Lipid from the wt. extracted by chloroform-ethanol	23-25	0-3	—	—
Phospholipid from the phosphorus content of the same extract	19	0.5-1	—	—

varies between 50 and 70 %, but most preparations contain 55-60 % of RNA and 40-45 % of protein.

For the remainder of the paper, the top layer of the sediment will be termed the 'phospholipo-protein fraction' and the bottom layer the 'mucoprotein fraction'. The free protein and RNA left after the ribosomes have been removed from the supernatant fraction will be termed the '5s-fraction of the complex'.

Electron microscopy of the fractions of the membrane complex. Under the electron microscope, the cytoplasm of sections of whole cells of *B. megaterium* is seen to contain abundant 150 Å particles, and to be surrounded by a double membrane underlying the cell wall (Plate 1a). Protoplasts present a similar picture, except for the absence of the cell wall.

The unwashed membrane complex obtained by conc. lysis of protoplasts (Plate 1b) is made up of a mixture of large, fairly empty vesicles bounded by a double membrane, smaller dense vesicles (in some sections, the preparation consists entirely of these smaller vesicles) and some strands that may represent DNA; 150 Å particles can again be seen, and are almost exclusively attached to the small dense vesicles. The phospholipoprotein fraction (Plate 1c) obtained by washing in dilute buffer the membrane complex prepared by conc. lysis consists entirely of very small membranous vesicles. These vesicles are smaller than any that are seen in the unwashed membrane complex and are often only 300-400 Å in diameter. No 150 Å particles are visible in this fraction. The well washed mucoprotein fraction (Plate 1d) is seen to consist of twisted irregular filaments, together with some fluffy diffuse material, and again no 150 Å particles are visible. Electron micrographs of isolated ribosomes show normal ribonucleoprotein particles.

Incorporation of [14 C]amino acids into components of the membrane complex

The membrane complex produced by conc. lysis of protoplasts can be actively incorporate [14 C]-amino acids into proteinaceous material when sus-

Fig. 3. Incorporation of L-[14 C]phenylalanine into the proteins of the components of the membrane complex after incubation of the isolated complex in 0.5M-phosphate-salts medium. Labelled amino acid (10 μ Ci) was added to membrane complex derived from 1.5 g. dry wt. of whole cells which were suspended at a final density of 2 mg./ml. Samples were taken at various times and fractionated and analysed as described in the Methods section. Δ , Phospholipoprotein; $\blacktriangle$, ribosomes; $\circ$, 5s protein; $\blacksquare$, mucoprotein.

pended in 0.5M-phosphate-salts medium under conditions described by Hunter *et al.* (1959). As mentioned above, the membrane complex obtained by dil. lysis is inactive in this respect. In some experiments, the isolated membrane complex was used for incorporation studies, but in most of the more recent work protoplasts were preferred as less manipulation was required to obtain the fractions.

The pattern of labelling of the isolated membrane complex during short exposures to [14 C]amino acids is illustrated in Fig. 3. In this experiment, the membrane complex was labelled with L-[14 C]-phenylalanine and samples were taken at 25, 80 and 360 sec. It can be seen that, at early times, the specific radioactivity of the protein of the phospholipoprotein fraction exceeds that found in the protein of the 5s-fraction of the complex, but, at later times, these specific activities are reversed. In

Plate 1. Electron micrographs of sections prepared as described by Mercer (1959) from: (a) whole cell of *Bacillus megaterium*; (b) a pellet of the membrane complex, prepared by conc. lysis of protoplasts, as described in the text; (c) the phospholipoprotein fraction; (d) the mucoprotein fraction; (c) and (d) were prepared by fractionating the membrane complex as described in the text.

longer incubations (up to 2 hr.), the specific radioactivity of the protein in the phospholipoprotein fraction tends to remain the same whereas that of the 5s-fraction continues to rise. At very early times (5–30 sec.) the specific radioactivity of the ribosomal protein (Figs. 3 and 4; Table 7) is either lower or about the same as that of the phospholipoprotein, a fact that has been confirmed in numerous experiments. The purity of the ribosomal fraction was checked by rerunning 30s and 45s particles from the density-gradient separation in the ultracentrifuge and also by determinations of the RNA:protein ratio. It is certain that the ribosome fraction obtained in these experiments is uncontaminated and made up only of 30s and 45s particles.

In the experiment illustrated in Figs. 4 and 5, protoplasts were labelled with L-[¹⁴C]threonine for 5 sec., 15 sec. and 2 min. The plot of the specific activities of the protein fractions (Fig. 4) shows that the course of labelling is similar to that observed in the isolated membrane complex, except that the highest specific activities eventually attained are now found in the cytoplasmic fraction. The plot of the same results in terms of total radioactivities (Fig. 5) illustrates well the fact that at

early times most of the label accumulates in the phospholipoprotein fraction and then seems to be passed on to the cytoplasmic fraction, possibly through the protein of the 5s-fraction of the complex. The total activity accumulating in the 5s-fraction of the complex is lower in protoplast experiments than in experiments with the isolated membrane complex, and some of the 5s-fraction probably consists of cytoplasmic precursors. There is less protein in the ribosomal than in the phospholipoprotein fraction and consequently the total radioactivity of the ribosomal protein is much lower than that of the phospholipoprotein fraction in a lot of this sort (Fig. 5). At all times, the labelling of the mucoprotein fraction is very low, even though it must be contaminated to a small extent by the phospholipoprotein fraction.

In summary, these experiments, which have been repeated many times with similar results, gave an indication that labelled protein, appearing first in the lipoprotein and ribosomal fractions, was passed on in sequence from the lipoprotein to the 5s-fraction of the complex and then to the cytoplasmic protein. The mucoprotein fraction, however, appeared to be inert. Further evidence in favour of this postulated sequence of events was provided by two types of 'chaser' experiments, in which the addition of the labelled amino acid to protoplasts was followed after a short time by a 'chase' of a large amount of the same unlabelled amino acid. Table 7 illustrates the changes in specific radioactivities of the various protein fractions when protoplasts were labelled for a very short time (10 sec.) and then 'chased' for 2 min. with a large amount of unlabelled amino acid. The specific radioactivity of the lipoprotein, initially relatively

Fig. 4. Incorporation of L-[¹⁴C]threonine into the protein of the cytoplasmic fraction and components of the membrane complex of protoplasts of *Bacillus megaterium*, suspended in 0.5M-phosphate-salts medium at a density of 50 mg./ml. Labelled amino acid (12 μc) was added to protoplasts derived from 1.5 g. dry wt. of whole cells. The samples were taken at various times and the fractions prepared and analysed as described in the Methods section. Δ, Phospholipoprotein; ▲, ribosomes; ○, 5s protein; ■, mucoprotein; ●, cytoplasmic fraction.

Fig. 5. Results of the experiment described in Fig. 4 plotted in terms of the total radioactivity present in each fraction. Δ, Phospholipoprotein; ▲, ribosomes; ○, 5s protein; ■, mucoprotein; ●, cytoplasmic fraction.

high, falls during the course of the 'chase', whereas the protein of the 5s-fraction and cytoplasm rises in activity. In this experiment, the specific radioactivities of both the 30s and 45s ribosomes was low. Table 8 illustrates an experiment where the 'chaser' dose was added after the incubation with the [^{14}C]amino acid had proceeded for 2 min., and the specific activities of the protein fractions were measured at intervals afterwards. Although the radioactivity in the lipoprotein fraction did not by any means drop to zero, it decreased progressively whereas the protein of the 5s-fraction of the complex and the cytoplasm gained a similar amount of label. In experiments of this type with rather longer times, the specific and total radioactivities found in the ribosomal protein are low.

Table 7. *Transfer of L-[^{14}C]phenylalanine incorporated into the protein of the components of the membrane complex and the cytoplasmic fraction of protoplasts of Bacillus megaterium*

B. megaterium were suspended in 0.5M-phosphate-salts medium after the addition of unlabelled L-[^{14}C]phenylalanine. Protoplasts, derived from 0.7 g. dry wt. of whole cells and suspended at a density of 40 mg./ml., were incubated with 0.15 μC of L-[^{14}C]amino acid for 10 sec. and then 16 mg. of 'chaser' L-[^{14}C]amino acid was added. For other details see Methods section.

Fraction	Specific activity after labelling for 10 sec. with	
	[^{14}C]amino acid ($\mu\text{mc/g.}$)	[^{14}C]amino acid followed by 'chasing' for 2 min. with [^{12}C]amino acid ($\mu\text{mc/g.}$)
Phospholipoprotein	0.194	0.11
5s-fraction	0.12	0.17
Cytoplasmic fraction	0.034	0.10
30s ribosomes	0.013	0.013
45s ribosomes	0.056	0.050

Table 8. *Transfer of L-[^{14}C]leucine incorporated into the protein of the components of the membrane complex and the cytoplasmic fraction of protoplasts of Bacillus megaterium*

B. megaterium were suspended in 0.5M-phosphate-salts medium, after the addition of L-[^{14}C]amino acid. Protoplasts, derived from 1.5 g. dry wt. of whole cells, were suspended at a density of 50 mg./ml. and incubated with 1.5 μC of L-[^{14}C]leucine for 2 min. and then 40 mg. of 'chaser' L-[^{12}C]leucine was added. For other details see Methods section.

Fraction	Total radioactivity (μmc)		
	2 min. after adding [^{14}C]leucine	4 min. after adding [^{12}C]leucine	12 min. after adding [^{12}C]leucine
Phospholipoprotein	15.0	12.3	9.5
5s-fraction	7.4	9.3	10.8
Cytoplasm	47	52	54
Ribosomes	0.53	0.53	0.53

DISCUSSION

The fractionation of the membrane complex described above enables its main constituents to be identified and their functions examined. Comparisons can also be made with observations on other cell types. It is now established that the membrane complex prepared by the lysis of protoplasts with comparatively concentrated salt solutions contains ribosomes, which can be detached by washing with buffers of low ionic strength. There is no conflict here with the findings of other workers (e.g. Tissières & Watson, 1958; McQuillen, Roberts & Britten, 1959), who have obtained ribosomes in cytoplasm. Their methods have all involved the use of media of low ionic strength, and in our experiments, also, these conditions lead to the appearance of ribosomal particles in the cytoplasmic fraction. In media of high ionic strength, ribosomes detached from the membrane complex are broken down, possibly because of the removal of magnesium ions. The ribosomes that remain attached to the membrane complex under these conditions are not degraded, perhaps because they are present in the more diffuse state suggested by Hanzon, Hermodson & Toschi (1959) to be the true physiological form of the ribosome. However, the electron micrographs of sections of both the whole cell and membrane complex show large numbers of particles 100–150 Å in diameter, and they can be obtained in the usual dense form after lysis. It is also remarkable that the activity of the membrane complex is maximal in a medium that would break down free ribosomes, presumably a further indication that the active ribosomes are bound in a special way to the membrane complex.

The solubilized extract containing the ribosomes also contains much material of 4–6s. This includes the amino acid-activation enzymes and also a quantity of RNA that is capable of binding amino acids in a form labile to the action of alkali and ribonuclease (Hunter *et al.* 1959). This RNA fraction probably corresponds to the soluble RNA fraction

of rat liver, but it is not clear at present whether it differs essentially from the cytoplasmic RNA removed previously.

The material that remains readily sedimentable after treating the membrane complex with dilute buffers is obtained in two layers which can easily be separated. The lower heavy layer or mucoprotein fraction contains little or no lipid or RNA and consists largely of carbohydrate with some protein. It appears mainly as large irregular fragments in electron micrographs. The phospholipoprotein fraction that lies above it also seems to be free from ribosomes, and indeed the low content of RNA, apart from the evidence derived from electron microscopy, would make it unlikely that an appreciable number could be present. It is not clear whether the phospholipoprotein fraction is derived entirely from the cytoplasmic membrane of the cell, or whether some of it originates in cytoplasmic structures associated with ribonucleoprotein. It is seen in the electron microscope as very small spherical vesicles often only 300–400 Å in diameter. The mucoprotein and phospholipoprotein fractions obtained by us are probably related to the fractions described by Weibull & Bergström (1958), although the experimental procedures outlined here enable the two fractions to be differentiated more clearly.

The data obtained when the membrane complex is fractionated after a preliminary incubation of the complex or protoplasts with labelled amino acids are rather complex. One fact stands out very clearly: the mucoprotein fraction is quite inert in the labelling process, confirming that our preparations were almost completely free from whole cells, since any whole cells present would have been obtained in this fraction. Labelled protein very soon appears in the 5s-fraction of the complex, but the results that have been obtained, and, in particular, the evidence of transfer of ^{14}C -labelled protein to this fraction obtained in the 'chaser' experiments, make it unlikely that any labelled protein is independently formed here. The primary site of formation of labelled protein thus seems to be in the phospholipoprotein fraction or in the ribosomes. Where the exposure to labelled amino acids has been carried out for short times only, the phospholipoprotein fraction always contains protein labelled at comparable or higher specific activity than that observed in the ribosomes. On the other hand, the total radioactivity in the protein of the phospholipoprotein fraction is considerably higher than that found in the ribosomes. If these ribonucleoprotein particles were the primary source of labelled protein and passed their nascent protein very rapidly on to the phospholipoprotein, then it would be expected that the 'chaser' experiments would show a very marked fall in the specific radioactivity of the ribosomes as their labelled nascent protein was re-

placed by practically non-radioactive material. However, the ribosomal protein was not observed to fall much in specific radioactivity during these experiments, and it is difficult to avoid the conclusion that the phospholipoprotein is labelled independently or concurrently.

Experiments carried out *in vivo* (e.g. McQuillen *et al.* 1959; Siekevitz & Palade, 1960) have been interpreted as indicating that the primary source of labelled protein is the ribosomal particle. However, the methods used by the latter workers for the preparation of the ribosomal particles were such that delicate phospholipoprotein structures would have been disrupted, and any freshly formed protein bound to them might have been released and attached to the ribosomes. The same criticism might apply in part also to the former group of workers (McQuillen *et al.* 1959), but in any case their results clearly show (especially their Fig. 6) that some material containing protein of high specific radioactivity sediments more rapidly than the ribosomes. This material may be the equivalent of our phospholipoprotein fraction, although most of the more readily sedimentable material was removed in their experiments and not examined at all. Where ribosomes have been obtained under conditions such that any associated phospholipoprotein would remain intact, they are often capable of very active protein biosynthesis (e.g. Webster, 1959). However, in the pea-seedling ribosome (Ts'o & Sato, 1958) the most highly labelled fraction again appears to be a phospholipoprotein. A ribosomal-free debris fraction has been obtained from *Escherichia coli* (Schachtschabel & Zillig, 1959; McCorquodale & Zillig, 1959) that is very active in incorporating amino acids into protein, and Dr P. N. Campbell (personal communication) has obtained evidence that serum albumin synthesis in rat-liver microsomes is located solely on the membrane part of the microsome.

There is thus considerable evidence for the implication of phospholipoprotein as well as the ribosomes in at least the final stages of protein biosynthesis. A possible mechanism to account for this has been suggested elsewhere (Hunter & Godson, 1961).

SUMMARY

1. The membrane complex obtained by lysing protoplasts of *Bacillus megaterium* in buffers of physiological strength is disrupted by exposure to buffers of low ionic strength.

2. Under these conditions, the following fractions are obtained and separated cleanly from each other: (1) a mucoprotein fraction; (2) a phospholipoprotein fraction, seen in the electron microscope as very small spherical vesicles free from ribosomes; (3) ribonucleoprotein particles (ribo-

somes of 30s and 45s); (4) ribonucleic acid of low molecular weight, together with solubilized protein that includes all the amino acid-activation enzymes of the complex.

3. When the whole membrane complex or protoplasts are incubated with labelled amino acids, labelled protein appears first in the ribosomal and phospholipoprotein fractions. The greater part of this labelled protein is found in the phospholipoprotein fraction, and there is no evidence that this is derived from the ribosomes.

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Enzymic Desulphation of L-Serine O[³⁵S]-sulphate: the Intracellular Localization of the Enzyme

By K. S. DODGSON AND N. TUDBALL

Department of Biochemistry, University College, Newport Road, Cardiff

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Dodgson, Lloyd & Tudball (1961*a, b*) reported that an enzyme system, capable of liberating inorganic [³⁵S]sulphate from L-serine O[³⁵S]-sulphate, is present in rat liver. The enzyme system could be extracted from fresh rat-liver suspensions with water. Studies on the localization of the enzyme system in the rat-liver cell and its distribution in other rat tissues and in other species have now been made.

MATERIALS AND METHODS

L-Serine O[³⁵S]-sulphate. This was prepared and diluted with carrier L-serine O-sulphate as described by Dodgson *et al.* (1961*b*).

Enzyme sources. Pig, sheep, chicken and ox livers and ox pancreas were slaughterhouse specimens. Human liver was a post-mortem sample provided by the Department of Pathology. Periwinkles (*Littorina littorea*) and limpets (*Patella vulgata*) were collected at low tide from the beach at Sully, Glamorgan. Snails (*Helix pomatia*) were purchased from L. Haig, Beambrook, Newdigate, Surrey. Brewer's yeast was kindly provided by Brain's Brewery, Cardiff. Salmon (*Salmo salar*) liver was obtained from the fish-market. Frogs (*Rana temporaria*) were kindly supplied by the Department of Zoology. Taka-diastase (lactose-free) was a gift from Parke, Davis and Co. Ltd., and acetone-dried cells of *Alcaligenes metalcaligenes* (see Dodgson, Melville, Spencer & Williams, 1954) were prepared at the M.R.C. Antibiotic Research Station, Clevedon.