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Intermediate Reactions in Protein Synthesis by the Isolated Cytoplasmic-Membrane Fraction of *Bacillus megaterium*

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Studies of the uptake of labelled amino acids into the various cellular fractions of *Bacillus megaterium* have shown that the primary site of protein synthesis in this organism is located in the fraction which sediments when broken protoplasts are centrifuged at 10 000 *g*. The activity probably resides in the principal component of this fraction, which is the cytoplasmic membrane (Hunter, Crathorn & Butler, 1957; Butler, Crathorn & Hunter, 1958). This may hold true for bacteria in general, as similar findings have now been reported for *Bacillus subtilis* (Nomura, Hosoda & Nishimura, 1958) and *Escherichia coli* (Spiegelman, 1959).

It was further shown (Butler *et al.* 1958) that the isolated cytoplasmic-membrane fraction was capable of incorporating labelled amino acids into proteinaceous material at rates comparable with those of the intact protoplast. Two aspects of the role of the isolated membrane system are considered here: (1) the relationship between normal protein synthesis in the whole cell and the incorporation of labelled amino acids into the cell-free system; (2) the incorporation process itself, with a view to identifying intermediate reactions. As previous studies of protein synthesis in other systems (Hoagland, Keller & Zamecnik, 1956; Hoagland, Stephenson, Hecht & Zamecnik, 1958; Nismann, Bergmann & Berg, 1957; De Moss & Novelli, 1955) have demonstrated the probable importance of amino acid-activating enzymes and of ribonucleic acid as factors controlling the course of amino acid incorporation into protein (so that peptides containing the amino acids can subsequently be obtained), particular emphasis has been laid on the investigation of their roles in this system.

A preliminary account of some of this work has already been given (Brookes, Crathorn & Hunter, 1959).

METHODS

Organism. The strain of *B. megaterium* (originally KM) was cultured as previously described (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 subcellular fractions. It was found that conversion of whole cells into protoplasts was best effected in a medium containing 0·02% of lysozyme and 0·5 M-KH_2PO_4, brought to pH 7·0 with NaOH. The lysozyme treatment was continued until a microscopic examination of a sample failed to reveal the presence of more than an occasional whole cell. The upper limit for whole-cell content was about 0·01%, although in many experiments not a single whole cell was observed in the protoplast preparations. Before carrying out any biochemical experiments, the protoplasts were usually aerated at 30° for 1 hr. in C medium containing, in addition, 0·5 M-KH_2PO_4 and 1% of glucose, the whole brought to pH 7·0 with NaOH. Lysis of the protoplasts was effected by diluting this medium with

1–2 vol. of water and shaking by hand until microscopic examination showed that no protoplasts remained intact. The cytoplasmic-membrane fraction was then collected by centrifuging at 10 000–20 000 *g* for 10–20 min.

Experiments with the isolated membrane fraction. Where the uptake of labelled amino acids was being studied, the cytoplasmic-membrane fraction was resuspended in C medium containing 0·5M-KH_2PO_4, the whole being brought to pH 7·0 with NaOH (C–phosphate). In short-term experiments, the presence of glucose had no effect on the system, but the addition of the 0·5M-KH_2PO_4 to the medium gave more reproducible results than the C–glucose used in earlier experiments (Butler *et al.* 1958). Many other media were tried, but all of these inactivated the amino acid-incorporation system to some extent, usually completely.

Further fractionation was effected by suspending membranes in water (25–100 mg./ml.) and subjecting the suspension to ultrasonic vibrations from a small MSE Mullard tissue disintegrator until it was completely homogeneous in appearance to the naked eye. Throughout this treatment, which usually lasted about 10 min., the tube containing the suspension was surrounded by a cooling bath so that the mean temperature in the tube did not rise above 30°. The sedimentable and 'soluble' fractions produced by this treatment were separated by centrifuging at 100 000 *g* for 5 min., and the ultrasonic treatment was repeated on the sedimented fraction. Homogeneity was more rapidly attained the second time. The homogenate was again centrifuged at 100 000 *g* and the 'soluble' fractions from the two successive treatments were combined.

Digestions with ribonuclease, lysozyme, histone and protamine (all at 1 mg./ml.) were carried out in C–phosphate, phosphate buffer or water.

Chloramphenicol was used at 100 μg./ml., and its inhibiting action was found to be complete after incubation at 30° for 15 min.

Digestions with 0·1N-NaOH were carried out at 30°.

Isolation of ribonucleic acid. Separation of the ribonucleic acid (RNA) from the 'soluble' fraction was effected by treatment of this fraction with an equal volume of aq. 90% phenol (Kirby, 1956). From the upper layer of this mixture phenol was extracted with ether. The aqueous extract was then brought to 2% with potassium acetate before precipitating the RNA with 2 vol. of ethanol. Contaminating carbohydrate was removed by extracting the RNA into 2-methoxyethanol (Kirby, 1956). After dialysis and drying in a vacuum desiccator, the RNA was obtained as an amorphous white powder. Some of the contaminating deoxyribonucleic acid (DNA) could be removed by dialysis against C medium (McQuillen, 1955) in the presence of deoxyribonuclease (10 μg./mg. of RNA), the purified RNA being recovered by precipitation with ethanol.

Protein solubilized in the course of the phenol extraction could be precipitated from the lower liquid phase by adding ether, removing the ether layer, sedimenting the precipitated protein and finally washing with ether.

Experiments on amino acid activation. These experiments were carried out under conditions similar to those used by Nismann *et al.* (1957). The medium contained 0·1M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), 5 mM-$MgCl_2$, 2 mM-adenosine triphosphate (ATP) and 5 mM-NaF. Solutions of amino acids to be examined were added to give a final concn. of 5 mM and the enzyme preparation was added to give 80 μg. of protein/ml. When the enzymic activities of cell cytoplasm or ultrasonically fractionated membranes were being investigated, they were prepared in the tris buffer as described above.

Incubations were carried out at 37° for 1 hr., and the ATP was finally 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 was determined.

Extraction of lipid fractions from membranes. Extractions with deoxycholate were carried out in a medium containing glycylglycine (2·64%), NaCl (1·17%) and sodium deoxycholate (0·5%), the whole being brought to pH 7·8 with NaOH. Membranes previously labelled with [^{14}C]amino acids were shaken in this medium (10 mg./ml.) at 37° for 20 min., followed by standing for 12 hr. at 0°. This procedure solubilized about 10% of the protein, the remaining 90% being removed by sedimenting at 20 000 *g*.

Extractions with methanol were carried out by boiling the appropriate fraction in the alcohol for 30 min. This same procedure was used whether the material extracted consisted of whole or fractionated membranes, proteinaceous fractions precipitated by trichloroacetic acid or material precipitated by the addition of solvents to phenol extracts.

Digestions with lipase were carried out under conditions similar to those used by Spiegelman, Aronson & Fitz-James (1958) when preparing 'nuclear bodies' from protoplasts. The membranes were digested at 30° at a concentration of 5–10 mg./ml.

Analysis of ^{14}C-labelled proteins. Samples of labelled protein (10–25 mg., containing 0·5–5·0 μc of ^{14}C/g.) from which lipids and nucleic acids had been removed as described previously (Crathorn & Hunter, 1957) were digested at 110° with 6N-HCl (0·2–0·5 ml.) for 18 hr. The HCl was removed, and about 10% of the sample was subjected to upward-flow chromatography on Whatman no. 1 paper in a solvent system containing methyl ethyl ketone–acetic acid–water (3:1:1, by vol.). Amino acid 'markers' were detected with a ninhydrin spray, and the papers were then placed in contact with X-ray film in a light-tight container for 2–4 weeks, and radioautographs were obtained in the usual way.

Partial hydrolyses of labelled samples (10–25 mg. at 2–5 μc of ^{14}C/g.) were carried out for 2 days at 37° in 12N-HCl (0·2–0·5 ml.). The acid was removed *in vacuo* over KOH and the residue dissolved in water (0·1 ml.). About one-half of the sample was subjected to two-dimensional paper chromatography (downward flow) in the following solvent systems: butanol–ethanol–propionic acid–water (10:5:2:5, by vol.) (solvent 1); butanol–acetone–di*cyclo*hexylamine–water (10:10:2:5, by vol.) (solvent 2). Papers were dried, sprayed with ninhydrin and radioautographs prepared as before, except that a longer time was generally required for their adequate exposure (4–8 weeks).

Assays of radioactivity. Protein and other solid samples were prepared for radioactive assay as described previously (Butler *et al.* 1958; Crathorn & Hunter, 1957). Assays of solid samples were 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 (supplied by 20th Century Electronics Ltd., New Addington, Surrey).

Table 1. *Effects of varying concentrations of suspended membranes on the uptake of* L-[^{14}C]*valine into protein fraction*

Membranes (100 mg.) were suspended in C–phosphate and shaken in 500 ml. flasks at 30° for 30 min. before addition of 2 μc of L-[^{14}C]valine. The incubation was continued in the same way for 20 min., and then brought to a close by adding aq. 30 % (w/v) trichloroacetic acid to a final concn. of 5 %. Other experimental details were as described in the text.

Concn. of suspended membrane (mg./ml.)	Specific radioactivity of protein (μc/g.)
1	0·20
5	0·45
20	0·12

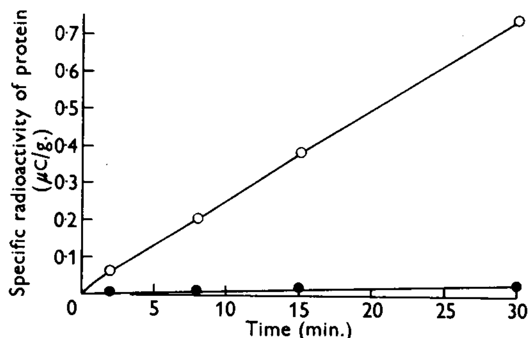

Fig. 1. Inhibition by chloramphenicol (100 μg./ml.) of the incorporation of [^{14}C]amino acids into the protein of the membrane fraction. Membrane samples (50 mg.) suspended in 'C–phosphate' (10 ml.) were shaken with chloramphenicol (100 μg./ml.) at 30° for 15 min. [1-^{14}C]-Glycine (0·2 μc) was then added to each sample and the incubations were continued for varying times before adding trichloroacetic acid. ○, Controls; ●, chloramphenicol present.

Estimation of nucleic acid. RNA was determined by the orcinol method (Cerriotti, 1955) and DNA by Cerriotti's (1952) indole method.

Materials. Generally-labelled L-amino acids (5–9 μc/μmole) and sodium [^{32}P]pyrophosphate were supplied by The Radiochemical Centre, Amersham, Bucks. [1-^{14}C]-Glycine (10·3 μc/mg.) was synthesized in these Laboratories. All the [^{14}C]amino acids were dissolved in water at 10 μc/ml. and used without any dilution with unlabelled amino acids, unless otherwise stated.

Lipase was supplied by the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; it was partially purified by the method of Spiegelman *et al.* (1958).

RESULTS

Experiments on the uptake of [^{14}C]*amino acids by the whole-membrane fraction.* Additional experiments (see also Butler *et al.* 1958) have enabled us to define more precisely the conditions required for maximum uptake of labelled amino acids. An improved medium has been developed and, further, it was found (Table 1) that maximum uptake of label into protein was achieved if the membranes were suspended in the medium at a concentration of 5 mg./ml. It was also found that the rates of amino acid incorporation were slightly enhanced if the membranes were thoroughly dispersed by shaking in a Mickle disintegrator rather than by hand. But the small advantage gained in this way was hardly sufficient to offset the extra manipulations involved.

The rates of uptake of different labelled amino acids have also been found to vary considerably, but as no information is available on the concentrations of amino acids present, both free and within the proteins, it has not been possible to make a quantitative assessment of these results.

Experiments with inhibitors. The effect of chloramphenicol on the incorporation system is illustrated in Fig. 1. After incubation for 30 min. in the presence of [1-^{14}C]glycine, the treated samples showed an inhibition of 96 % in the uptake of the labelled amino acid into the protein fraction. In a corresponding experiment with whole cells carried out at the same time, the uptake of [1-^{14}C]glycine into the fraction precipitable by trichloroacetic acid was five times greater than that in the membrane experiment, but the inhibition produced by the same concentration of chloramphenicol was again 96 %.

Table 2. *Effect of pre-incubating the membrane fraction with ribonuclease and other proteins on the amino acid-incorporation system*

For each treatment, membranes (50 mg.) were suspended in C–phosphate (10 ml.) containing the dissolved protein (10 mg.). After shaking at 30° for 1 hr., L-[^{14}C]lysine (2 μc each) was added to one-half of the reaction flasks and the incubations were continued for a further 30 min. The membranes were sedimented and resuspended in fresh medium before L-[^{14}C]lysine was added to the other flasks. The incubations were terminated and the protein samples obtained as described in the text.

	Specific radioactivity of membrane protein (μc/g.)	
Protein added	Original medium	Fresh medium
—	0·52	0·63
Ribonuclease	0·033	0·29
Lysozyme	0·050	0·32
Histone	0·012	0·053
Protamine	0·008	0·112

Table 2 shows the effect produced on the incorporation system by pre-incubating the membranes with ribonuclease and various basic proteins.

The reversible nature of the inhibition produced by ribonuclease under conditions where it is enzymically active, and the similarity to that produced, in particular, by lysozyme would suggest that it is not a consequence of its action as an enzyme.

Total and partial acid hydrolysis of labelled protein from membranes. Total hydrolyses were carried out on protein samples derived from membranes that had been labelled with [1-^{14}C]glycine, L-[^{14}C]lysine and L-[^{14}C]valine. In each case, radioautographs of the hydrolysates showed that the original amino acid added was the only detectable radioactive compound present. However, other compounds containing less than 10% of this radioactivity might have remained undetected. Partial acid hydrolyses were carried out on membranes labelled with L-[^{14}C]leucine, L-[^{14}C]valine and L-[^{14}C]-alanine. In each case the radioautographs showed that several labelled peptides were present in the hydrolysates. The original amino acid was also present, but there appeared to be a larger proportion of the label in the peptide spots (Fig. 2).

Experiments with ultrasonically disrupted membranes. When membranes labelled for 20–30 min. were disrupted ultrasonically, it was found that the protein of the sedimented fraction had about one-sixth of the specific radioactivity of the protein of the 'soluble' fraction. Thus membranes (0·5 g.) labelled for 30 min. with L-[^{14}C]valine (10 μc) in the usual way gave 'soluble' protein with a specific radioactivity of 2·6 μc/g. and sedimentable protein with a specific radioactivity of 0·41 μc/g. When the labelling time was reduced, the specific radioactivity of the sedimentable protein was relatively higher; indeed, when the time was only 2 min. the two protein fractions had comparable specific radioactivities.

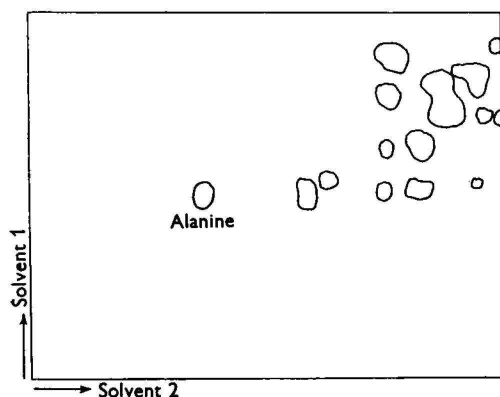

Fig. 2. Tracing of radioautograph prepared from two-dimensional paper chromatogram of partial acid hydrolysate of protein from membrane fraction labelled with L-alanine. All the operations were carried out as described in the text. All the areas outlined represent peptides, apart from the L-alanine spot itself.

The ultrasonic treatment was also carried out on unlabelled membranes suspended in C–phosphate, and the fractions obtained were separately incubated with L-[^{14}C]alanine. As shown in Table 3, only the sedimentable protein could be labelled directly, although the level of labelling obtained was much lower than that attained with whole membranes.

Experiments on amino acid activation. Initial experiments were performed on whole membranes, rather variable results being obtained. Exchange of ^{32}P between [^{32}P]pyrophosphate and ATP was, however, found to take place at rates 100–300% above control values with the following amino acids: L-leucine, L-proline, L-asparagine, L-cysteine, L-lysine and L-histidine. Chloramphenicol at 100 μg./ml. had no effect on the process. No significant enzymic activities were observed when equivalent amounts of cytoplasm were used.

However, when the membranes were suspended in the tris buffer used for the ^{32}P-exchange experiments and disintegrated ultrasonically, it was

Table 3. *Separate labelling of fractions derived ultrasonically from whole membranes*

Membranes were fractionated as described in the text. Fractions (50 mg.) were adjusted to 5 mg./ml. in C–phosphate and then shaken at 30° with L-[^{14}C]alanine (2 μc) for 30 min., before adding trichloroacetic acid to a final concentration of 5%.

Fraction	Specific radioactivity of protein (μc/g.)
Whole membranes	5·0
Sedimented fraction	0·11
'Soluble' fraction	0·005

Table 4. *Transfer of* ^{32}P *from sodium* [^{32}P]*pyrophosphate to adenosine triphosphate in the presence of the 'soluble' fraction of ultrasonically disintegrated membranes*

Preparation of the 'soluble' fraction and the conditions of the ^{32}P-exchange reaction were as described in the text. For each treatment, 0·25 μc of sodium [^{32}P]pyrophosphate (specific activity 6·40 mc/m-mole) was used in a total volume of 2 ml.

Treatment	Percentage transfer of ^{32}P to ATP
Control, without 'soluble' fraction	0·4
Control, without added amino acid	1·9
Control, without added ATP	0·8
L-Asparagine	6·0
L-Cysteine	11·0
L-Histidine	6·3
L-Leucine	14·8
L-Lysine	11·0

found that the amino acid-activation enzymes were liberated into the 'soluble' fraction. All the amino acids studied stimulated the rate of ^{32}P-exchange between pyrophosphate and ATP several times above the control values (Table 4). Very little of the enzymic activity resided in the sedimented fraction.

Properties of ribonucleic acid from the membrane fraction. In a typical experiment, 2·5 g. of whole cells were lysed in the usual way and phenol extraction of the ultrasonically disrupted membrane fraction was carried out as described in the Methods section. The crude RNA had $\lambda_{max.}$ 260, $E_{1\,cm.}^{1\%}$ 30, which after purification gave 30 mg. of solid having $\lambda_{max.}$ 260, $E_{1\,cm.}^{1\%}$ 210. The final product was completely soluble in hot trichloroacetic acid. Analyses indicated that the material was about 70% of RNA and 30% of DNA. After treatment with deoxyribonuclease, the RNA content rose to 87% (colorimetric analysis). The product contained 6·2% of P, and a base ratio determination (Wyatt, 1951) showed that thymine was absent.

Where the membranes had been pre-incubated with [^{14}C]amino acids, the RNA obtained carried a radioactive label. The amount of the label varied widely from one experiment to another. Thus when 500 mg. of membranes were incubated for 20 min. with 10 μc of labelled amino acids under the usual conditions, the specific radioactivities of the purified RNA obtained in six different experiments varied from 0·03 to 5·0 μc/g. The yields of RNA obtained also varied between 5 and 25 mg., usually being less than the yield obtained when freshly prepared membranes were extracted immediately without any incubation at 30°.

The yields of RNA (by wt.), as well as the degree of labelling, bore no obvious relation to the specific radioactivity of the protein obtained in the same experiments. But this may be due to technical deficiencies in the method of RNA extraction. Table 5 shows the results of one such experiment in which the final specific radioactivity of the protein was 0·6 μc/g.

Table 5. *Labelling of membrane ribonucleic acid with* L-[^{14}C]*alanine*

The membranes (540 mg.) were incubated as described in the text with 10 μc of L-[^{14}C]alanine. One-third of the suspension was withdrawn at each of the indicated times, cooled rapidly to 0° and further treated as described in the text.

Incubation time (min.)	Specific radioactivity of RNA (μc/g.)
2·5	0·018
7·5	0·16
20	0·48

It does not seem likely that any appreciable quantity of the label attached to the RNA can be ascribed to metabolic conversion of the added amino acids into purines or pyrimidines. Thus labelled RNA (0·12 μc/g.) obtained in one experiment where L-[^{14}C]arginine was used was subjected to acid hydrolysis. 'Carrier' unlabelled L-arginine was added to the hydrolysate and found to retain the bulk of the ^{14}C label on recrystallization. However, the purified samples of RNA appeared to be free from labelled protein: precipitation with cold trichloroacetic acid in the presence of a large excess of unlabelled protein, followed by heating with hot trichloroacetic acid, left a residue that contained no radioactivity. On the other hand, the radioactivity could readily be removed by mild treatments at alkaline pH. Thus labelled RNA (0·2 μc/g.) derived from membranes incubated with L-[^{14}C]valine lost 30% of its radioactivity when left at room temperature for 10 min. in 0·02N-KOH, and 90% if the temperature was raised to 30° for the same period, the RNA being recovered in each case by precipitation with ethanol. Of the radioactivity, 75% was removed by treatment with ribonuclease (1 mg./ml.) for 1 hr. at 30°. Treatment with deoxyribonuclease, on the other hand, had no effect.

In one experiment where labelled RNA (10 mg., 0·2 μc/g.) was incubated for 10 min. in C–phosphate (10 ml.) with the sedimented fraction (10 mg.) derived from ultrasonically treated membranes, 37% of the label was transferred to material insoluble in hot trichloroacetic acid. Guanosine triphosphate (0·1 mg./ml.) was added to the normal C–phosphate medium. Attempts to repeat this experiment have been successful in only one out of four, and similar 'transfer' experiments carried out with whole membranes have also given negative results. However, a 10% transfer of the label from RNA to the protein of whole protoplasts has been effected, and experiments are being continued.

Experiments suggesting a role for lipids on protein synthesis. Treatment of labelled membranes with sodium deoxycholate led to the solubilization of about 10% of the protein. The radioactive materials in both soluble and insoluble fractions were still precipitable by hot trichloroacetic acid, but, as shown in Fig. 3, the specific activity of the protein in the extracted lipoprotein fraction was much the higher, whatever the length of time for which the membranes were labelled.

This result led us to examine the action of lipase on labelled membranes. Many experiments were performed, with somewhat variable results, but it was always found that incubation in the presence of lipase resulted in a rapid loss of radioactivity from the fraction insoluble in hot trichloroacetic

acid. This is illustrated in Fig. 4. In the course of the same experiment, the weight of material recovered in the protein fraction dropped to 50% of the control in the first 5 min. of the lipase treatment, but thereafter fell very little more in 55 min. In further experiments it was found that a greater percentage of the radioactivity could be removed from membranes labelled for short periods than for periods of 30 min. or more.

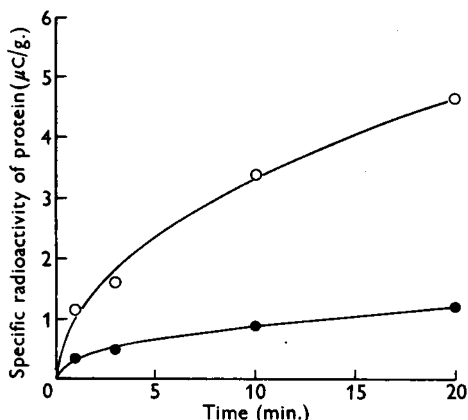

Fig. 3. Deoxycholate fractionation of membrane protein labelled with L-[^{14}C]leucine. The membrane fraction was labelled for varying times as described in the text. ●, Residue from extraction with sodium deoxycholate; ○, fraction extracted during treatment with sodium deoxycholate.

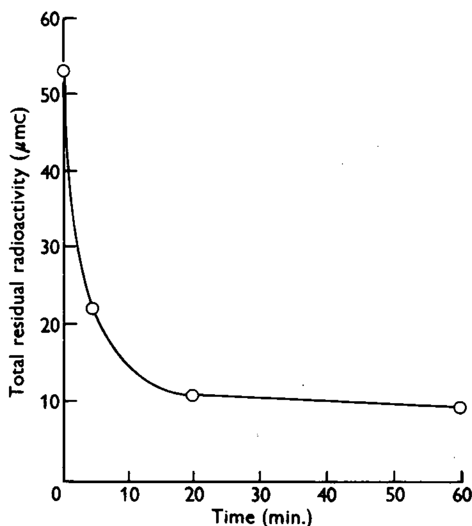

Fig. 4. Rate of removal of radioactivity by lipase from the protein fractions of membranes labelled in the usual way with L-[^{14}C]lysine. In each treatment, membranes (40 mg.) were treated with crude lipase (10 mg.) under the conditions described in the text.

It was also found that incubation of labelled membranes with 0·1 N-NaOH at 30° for 10 min. removed between 10 and 50% of the protein-bound radioactivity.

Finally, the extraction of labelled membranes with boiling methanol led to the removal of considerable amounts of radioactivity in a form no longer precipitable with trichloroacetic acid. The bulk of the methanol-soluble material was present in the part of the membrane fraction which was still sedimentable after the ultrasonic treatment (see above). As shown in Table 6, about 62% of the label in this fraction was extracted into methanol. Preliminary chromatographic results indicate that the material extracted is not in the form of the free amino acid; its composition is probably complex and it is being examined in greater detail.

However, the whole of the label extracted in this and in the other experiments described in this section cannot be attributed to common lipid constituents formed by metabolic processes from the amino acids. Extraction with lipid solvents at temperatures below 40°, although it solubilizes much of the lipid, fails to lower the specific radioactivity of the protein; the label is therefore not present in the common lipids. Only treatments such as boiling with methanol, which is reported to disrupt lipoprotein complexes (Reichert, 1944), liberates the label and it might be inferred that the label is present in such a form. Furthermore, since total hydrolysis of the whole membrane fraction enabled the bulk of the activity to be recovered in the form of the original amino acid, it can be concluded that the methanol extract also contains the amino acid residue in some form.

DISCUSSION

The present investigation has shown that the isolated cytoplasmic-membrane fraction of *B. megaterium* is capable of incorporating labelled amino acids into peptides. Since (1), the incorporated [^{14}C]amino acid can be retrieved unchanged from total hydrolysates of the labelled-protein fractions under conditions where no other [^{14}C]-compound is detected, and (2) numerous labelled

Table 6. *Removal of bound radioactivity from membrane fractions by extraction with methanol*

Membranes (500 mg.) labelled with L-[^{14}C]phenylalanine (10 μc) for 15 min. were fractionated as described in the text.

	Specific radioactivity of protein (μc/g.)	
Fraction	Before methanol extraction	After methanol extraction
Sedimented	0·20	0·074
'Soluble'	0·52	0·51

peptides are present in partial hydrolysates of [^{14}C]protein, it is concluded that some of the amino acid must have been bound in peptide form, and probably in large peptides or proteins. The fact that the incorporation process is affected by chloramphenicol to the same extent as protein synthesis in intact cells further indicates that the normal mechanisms of protein synthesis are operating in the isolated membranes. Although we cannot completely rule out the presence of whole cells in all our membrane preparations, they were not observed in several experiments where fully active membrane preparations were obtained. In any case, the maximum contamination of 0·01 % would appear to be grossly inadequate to account for the incorporations of labelled amino acids that have been observed.

With the procedure adopted for extracting, washing and assaying the radioactivities of protein fractions, adsorption errors appear to be very small. If labelled-protein samples are dialysed against aqueous solutions containing large amounts of the corresponding unlabelled amino acid before or after the addition of trichloroacetic acid, the final counts obtained are not significantly altered. For a time, precipitations with trichloroacetic acid were carried out in the presence of excess of the unlabelled amino acid, but as several controlled experiments showed that this procedure, also, made no significant difference it was discontinued.

The membrane fraction appears to contain all of the amino acid-activating enzymes of the cell, and these are liberated in a soluble form by ultrasonic treatment. It is possible that activation enzymes for all the common amino acids are present, as in the *Escherichia coli* preparation of Nismann *et al.* (1957).

The successful isolation of RNA from the membrane fraction suggests that Weibull & Bergström (1958) may be wrong to regard as insignificant the 1–2 % of RNA that they detected in the cytoplasmic membrane of *B. megaterium* strain M. It has been shown here that RNA from the membrane fraction can take up labelled amino acids in an alkali-labile form, and this is probably an intermediate of protein synthesis as in the mammalian cell (Hoagland *et al.* 1958). On the other hand, we have not detected any appreciable binding of [^{14}C]amino acids to cytoplasmic RNA in tracer experiments with protoplasts, although the RNA from the membrane fraction in the same experiments has been labelled. Thus in strain KM at least the 1–2 % of RNA is probably an integral part of the membrane fraction. However, we have not obtained conclusive evidence that this bound amino acid can be transferred to the protein of the membranes. The rate of labelling of the RNA does not seem to be related in any way to the rate of labelling of the protein, but too little is at present known about the intermediate stages of protein synthesis to draw any definite conclusions. It has been shown (Cohn, 1959) that fractions of rat-liver microsomes can incorporate labelled amino acids efficiently into their protein fractions in the absence of detectable amounts of the pH 5·0 enzyme system, so that here, too, the role of RNA–amino acid complexes must remain open for the time being. It is unlikely that the cytoplasmic RNA of *B. megaterium* plays any direct part in protein synthesis. We have found (unpublished work) that prolonged centrifuging (20 hr. at 100 000 *g*) of cytoplasm derived from lysed protoplasts leads to the sedimentation of particles containing the bulk of the RNA, but tracer experiments with [^{14}C]amino acids have shown that these particles always contain protein of lower specific radioactivity than any other cellular fraction.

The results of the ultrasonic fractionation show that the labelling of the protein in the membrane fraction is far from uniform. At first it appeared that the solubilized fraction was the all-important one, especially when it was found that it contained the enzymes concerned with amino acid activation, and also amino acids bound to RNA. However, it was soon found that the isolated sedimentable fraction can incorporate labelled amino acids into protein whereas the 'soluble' fraction cannot do so (Table 3). This finding, coupled with the fact that short-term labelling experiments on whole membranes give [^{14}C]protein distributed equally between the two fractions, would seem to indicate that both fractions are necessary for full activity of the system. The small activity shown by the sedimentable fraction in isolation may well depend upon the presence of traces of the 'soluble' fraction for the preliminary activation of added amino acids.

Finally, evidence has been given that a further factor or factors may play a role in the incorporation process, and these factors appear to be associated with a lipid fraction. In contrast with results obtained in the rat-liver-microsome system (Littlefield, Keller, Gross & Zamecnik, 1955), extraction of labelled membranes with deoxycholate solubilizes a 'lipoprotein' fraction containing protein of much higher specific radioactivity than the residual protein. It has not yet been determined whether this 'lipoprotein' fraction corresponds to the fraction of the labelled protein solubilized by treating the membranes with lipase; we have not had available lipase completely free from proteolytic enzymes. However, it seems unlikely that the results obtained could be attributed entirely to the latter action. Prolonged digestion of labelled membranes with chymotrypsin in fact failed to remove more than a small fraction of the [^{14}C]-protein. The material removed by lipase is no

longer precipitable by trichloroacetic acid; this also applies to the bulk of the labelled compounds extracted by boiling methanol. A further chromatographic examination of the methanol extracts is being made. These results make it seem likely that lipids play a part in protein synthesis, and indeed the cytoplasmic membrane of *B. megaterium* has a high lipid content (Weibull & Bergström, 1958). Hendler (1958) has produced similar strong evidence for the existence of lipid intermediates in protein synthesis in the hen-oviduct system. If both RNA and lipids are concerned in protein synthesis, then our results would indicate that the lipids are involved at a stage subsequent to the action of RNA.

SUMMARY

1. A study has been made of the conditions necessary to obtain high and reproducible levels of incorporation of [^{14}C]amino acids into the protein fraction of the isolated cytoplasmic membrane of *Bacillus megaterium*.

2. Degradation of the labelled protein obtained has been carried out by partial and total acid hydrolysis. It was found that in all the cases investigated the added [^{14}C]amino acid had been incorporated unchanged into peptides.

3. Peptide-bond formation in the membrane system was inhibited by chloramphenicol to exactly the same extent as protein synthesis in the whole cell.

4. The cytoplasmic-membrane fraction contained amino acid-dependent enzymes catalysing the exchange of ^{32}P between [^{32}P]pyrophosphate and adenosine triphosphate. These enzymes are not inhibited by chloramphenicol.

5. The membrane fraction may be split into two fractions (soluble and sedimented) by treatment with ultrasonic vibrations. The soluble fraction contains the amino acid-activating enzymes and also the ribonucleic acid in an extractable form.

6. When the membranes are labelled with [^{14}C]-amino acids, the amount of radioactivity in the protein of the soluble fraction increases much more rapidly with time than that of the sedimented fraction. The ribonucleic acid of the soluble fraction carries bound radioactivity in a form not precipitable with hot trichloroacetic acid. It is readily removed by mild treatments at alkaline pH or by ribonuclease.

7. The possible role of the ribonucleic acid in the amino acid-incorporation process is discussed. Evidence has also been obtained indicating that lipids may play a part in protein synthesis.

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