The present work shows that a soluble degradation product of elastin can be converted into an insoluble form, having many of the characteristic properties of the original elastin. The way in which elastin is formed *in vivo* is not known with certainty but it is at least a possibility that it is formed from a soluble precursor by a mechanism similar to reconstitution.

SUMMARY

1. The high-molecular-weight α -fraction of soluble elastin prepared from ox *ligamentum nuchae* (Adair *et al.* 1951) becomes insoluble on prolonged heating of aqueous solutions under certain conditions.

2. Heat-precipitated elastin has many of the physical properties of purified elastin and it is concluded that the protein molecules have essentially the same configuration in the two materials.

3. Differences between heat-precipitated (reconstituted) elastin and purified elastin may be attributed to degradation of the latter when it is converted into soluble elastin.

4. The precipitation of elastin appears to consist of two processes, namely a rapid one which may involve configurational changes and results in coacervation and a slow one which is probably due to aggregation of the protein molecules.

5. The bearing of the results on the structure of elastin and its fibrogenesis is discussed.

I wish to thank Miss H. Saxl for studying the histology of purified and precipitated elastin and Dr W. G. Horton for examining precipitated elastin by X-ray diffraction. I also wish to thank Miss H. Saxl, Dr D. A. Hall and Professor R. E. Tunbridge for helpful discussion and criticism and Miss J. Waddington for technical assistance.

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The Site of Protein Synthesis in *Bacillus megaterium*

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In recent years much work has been done on the incorporation of isotopically labelled amino acids into the various fractions of mammalian cells. It has been found that the ribonucleoprotein particles associated with the microsomal fraction become labelled at a very early stage (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Keller, Zamecnik & Lofffield, 1954; Littlefield, Keller, Gross & Zamecnik, 1955; Simkin & Work, 1957;

Cohn & Butler, 1957), although evidence for a preliminary activation process in the 'soluble fraction' has been given (Hoagland, Keller & Zamecnik, 1956; Hoagland, Zamecnik & Stephenson, 1957). On the other hand, little information has been obtained about the sites of protein synthesis in the bacterial cell. It has, however, been shown that in *Torulopsis utilis* absorbed amino acids do not exist in the free state at all but are bound in such a way

that they are not freely exchangeable with amino acids in the surrounding medium (Cowie & Walton, 1956), and Gale & Folkes (1955) found that a cell-envelope fraction from *Staphylococcus aureus* was capable of developing enzyme activities and of incorporating labelled amino acids into protein. These two reports indicated that the cell wall or cytoplasmic membrane might play an important part in the synthesis of protein by micro-organisms.

Bacillus megaterium seemed to us a suitable micro-organism for studies of the site of protein synthesis in view of the work of Weibull (1953a, 1955). He had shown that it is easily possible to remove the cell wall by treatment with lysozyme, leaving, when suitable precautions are taken, the protoplasm of the cell in the form of spherical protoplasts.

It has been shown (McQuillen, 1956) that these protoplasts are able to synthesize proteins, nucleic acids and enzymes, including adaptive enzymes; they can also grow and divide and allow the development of endospores. When shaken violently, or placed in media of low osmotic pressure, they can be lysed, with the resultant liberation into the medium of the soluble cell constituents. Two structures then remain: empty vesicles or 'ghosts' derived from the cytoplasmic membrane that originally surrounded the protoplasts and small granules. The membranes and granules may be isolated by differential centrifuging (Weibull, 1953b), and the separated granules appear to consist largely of polymerized β -hydroxybutyric acid. If the membrane-granule fraction is sedimented under the conditions described in this paper, the clear supernatant fraction (cytoplasm) deposits little further sediment when centrifuged for 30 min. at 100 000 g, so that there appears to be no close equivalent of the mammalian microsome fraction present (Hunter, Crathorn & Butler, 1957).

In the experiments reported here a study has been made of the incorporation of labelled amino acids into the cell wall, the cytoplasmic membrane and the supernatant fractions. The incorporation has been studied in (1) the intact bacterium; (2) intact and broken protoplasts; (3) the membrane and supernatant fractions of the protoplasts. A preliminary account of part of this work has been given (Crathorn & Hunter, 1958).

METHODS

Organism. We are indebted to Dr K. McQuillen for supplying the original strain KM of *Bacillus megaterium*. It has been maintained by frequent subculture on peptone-agar slopes. By progressive selection, a strain has been isolated that forms very stable protoplasts when treated with lysozyme. These can be treated almost as whole cells

when suspended in neutral media containing 0.5 M-phosphate and can then be disintegrated only after very violent shaking.

The organism was usually grown at 30° in a glucose-salts solution (McQuillen, 1955) medium, although on some occasions the glucose was replaced by 0.5% (w/v) peptone (British Drug Houses Ltd., Poole, England) without apparently affecting any of the biochemical properties studied here. Large batches for experimental purposes were prepared in aerated Roux bottles or 5 l. shake flasks.

Experiments with whole cells. Whole cells from large batches grown overnight were harvested and washed, suspension densities being determined from the absorption at 420 m μ on a Unicam spectrophotometer (model SP. 600) previously calibrated against the organism. For tracer experiments the cells were resuspended in the glucose-salts medium and re-incubated aerobically at 30° for 1 hr. before adding any [¹⁴C]amino acid. After further aerobic incubation for the required length of time, the cells were sedimented and resuspended in a medium containing 0.5 M-sodium citrate, 0.5% (w/v) of casein digest (Bacto-Casamino acids; Difco Laboratories Inc., Detroit, Mich., U.S.A.), and 0.02% of lysozyme. Protoplasts were collected by centrifuging after standing overnight at 2°, and the digested cell-wall material was obtained in the supernatant medium. The protoplasts were finally lysed by pouring into 4 vol. of water and the fraction containing the cytoplasmic membrane and granules was collected by sedimentation at 10 000 g for 10 min. In some experiments it was found necessary to sediment at 20 000 g for 20 min. to obtain a firm pellet.

The supernatant fraction from this last centrifuging contained the cytoplasm of the cell diluted with the suspension medium. It was found that estimations of the radioactivity of protein in this fraction were in many cases little affected if the cell-wall digest and free amino acids were also present, and in some experiments a better control of timing was attained by pouring the original cell suspension directly on to ice at the end of the period of incubation with the labelled amino acid. Lysozyme was added to a final concentration of 0.02% and the cytoplasmic membranes were isolated as before after standing overnight at 2°.

In some cases the cytoplasmic-membrane fractions were further treated with ethylenediaminetetra-acetic acid (EDTA) (1% sodium salt; pH 7) at 30° for 1 hr. This had the effect of solubilizing many of the granules and bringing into solution about one-third of the protein and some of the deoxyribonucleic acid (DNA). The actual amount of DNA solubilized in this way was variable (35–65%). Vennes & Gerhardt (1956) claim that it is possible to remove DNA completely from the cytoplasmic membranes of *B. megaterium* by treating protoplasts with a mixture of EDTA and deoxyribonuclease, but under conditions where the activity of the enzyme was probably very small.

Experiments with protoplasts. Protoplasts were prepared by gently shaking suspensions of whole cells at a density of 1 mg./ml. (dry wt.) in air at 30° in a medium containing 0.5 M-sodium citrate, 0.5% of casein digest and 0.02% of lysozyme. The complete conversion into protoplasts usually took about half an hour under these conditions, sometimes much longer. On occasion, suspensions were obtained that were partially or completely insensitive to the action of lysozyme under these conditions. We can offer no

explanation of this phenomenon; fortunately it is not of very frequent occurrence. The protoplasts were sedimented and resuspended for incorporation experiments in 'C' solution (McQuillen, 1955) containing, in addition, 0.5M KH_2PO_4 and 1% of glucose, the whole brought to pH 7 with sodium hydroxide. In this medium they were very stable and could be shaken vigorously enough to maintain adequate aeration without appreciable lysis taking place. It is always advisable, however, to follow closely by microscopic examination, any metabolic experiments with whole protoplasts.

After shaking at 30° in this medium for 1 hr. labelled amino acids were added and any further period of incubation of the protoplasts could be defined clearly by pouring the whole or part of the suspension directly on to 4 vol. of ice-water, when instantaneous lysis occurred. The cytoplasmic membrane fraction was then separated at once from the cytoplasmic fraction by centrifuging at 10 000–20 000 g for 10–20 min.

Experiments with broken protoplasts and isolated membranes. Thick suspensions of protoplasts (20 mg./ml.) in phosphate-'C'-glucose medium, detailed in the previous section, were broken by rapid oscillation in a Mickle tissue disintegrator. The suspension was then diluted 20-fold with the same medium and incubated aerobically at 30° for 30 min. ^{14}C -Labelled amino acid was then added in order to study the incorporation of the amino acid into protein and the incubation continued for varying times, eventually being stopped by rapid cooling. The membrane fraction was then separated from the cytoplasm and incubation medium by sedimentation at 20 000 g for 20 min.

In other experiments, the cytoplasmic membranes were separated from the cytoplasm by centrifuging and the incorporation of [^{14}C]amino acid was studied in the two separated fractions. The protoplasts could also be broken by diluting twice with water and shaking vigorously by hand for 2 min., and the properties of the isolated membranes and cytoplasm thus obtained were very similar to those of the corresponding fractions obtained by the previous treatment. For incorporation studies on the isolated membranes, they were usually resuspended in the inorganic 'C' solution with 1% of glucose added.

When transfer experiments were carried out with cytoplasmic membranes labelled *in vitro*, the labelling medium was removed and the washed membranes were then re-incubated aerobically with non-labelled cytoplasm at 30° for the appropriate period of time. The cytoplasm and the membranes were then separated once more by centrifuging at 20 000 g for 20 min.

Attempts to obtain incorporation of [^{14}C]amino acids into the protein of isolated cytoplasm were made by incubating the cytoplasm aerobically at 30° in the presence of various additives.

Estimation of nucleic acids. Ribonucleic acid (RNA) was determined in whole cells and subcellular fractions by the orcinol method (Cerriotti, 1955). DNA was determined by the indole method (Cerriotti, 1952). Samples of DNA and RNA which had been prepared from whole cells by a detergent method and separated by differential centrifuging (Hunter & Butler, 1956) were used as standard. These separated nucleic acids also served as standards in estimating the proportions of RNA and DNA in mixed nucleic acids extracted by treatment of various fractions with sodium dodecyl sulphate. Base analyses of extracted

nucleic acids (Wyatt, 1951) were carried out after hydrolysis with perchloric acid (Marshak & Vogel, 1951).

Preparation of samples for radioactivity assay. Protein samples for counting were obtained from suspensions or solutions by adding aqueous trichloroacetic acid to a final concentration of 5% (w/v), and then removing lipids and nucleic acids from the precipitated proteins as described previously (Crathorn & Hunter, 1957). Where the total radioactivity of a given fraction was required it was evaporated to dryness at 90° and counted directly.

Assays of radioactivity. These were carried out where possible on 'infinitely thick' samples of protein or dried bacterial fractions plated on 1 cm.² polythene disks (Popják, 1950), an EHM2S end-window Geiger-Müller counter in conjunction with an N529 scaling unit (manufactured by Ekco Electronics Ltd., Southend-on-Sea, Essex) being used. The counter was housed in a lead castle manufactured by E.R.D. Ltd., Slough, Bucks. Under these conditions a ^{14}C -labelled protein sample of specific activity 1 $\mu\text{C/g.}$ would give a counting rate of about 500 counts/min. when plated at 30 mg./cm.² (i.e. an efficiency of 1.2%). Samples of less than 'infinite' thickness were plated uniformly in the disks, and the counts obtained corrected to 'infinite' thickness by making use of an experimentally determined curve obtained with bacterial protein of known specific activity.

Materials. Generally labelled L-amino acids were supplied by the Radiochemical Centre, Amersham, Bucks. [^{14}C]Glycine (10.3 $\mu\text{C/mg.}$) and DL-[^{14}C]phenylalanine (12.0 $\mu\text{C/mg.}$) were synthesized in these Laboratories by Dr P. Brookes and Dr V. C. E. Burnop. All the [^{14}C]amino acids were used throughout this work without any dilution with unlabelled amino acid.

RESULTS

Experiments with whole cells. In order to determine the extent to which the cell wall participated in incorporation of amino acid, various ^{14}C -labelled amino acids were added to whole cells of *B. megaterium* growing in the full glucose-salts medium and incubations were continued for 5 or 30 min. before removing the cell walls with lysozyme. The results are summarized in Table 1; it can be seen that the specific activity of the cell-wall 'protein' is much lower than that of the protoplast protein in all cases. Of course, this might not have been true if any of the three amino acids most abundant in the cell wall of *B. megaterium* (alanine, glutamic acid and diaminopimelic acid) had been used. But it showed that the cell wall played no major part in the early stages of the synthesis of the bulk of the protein of the cell.

The rate of incorporation of ^{14}C -labelled amino acids into the proteins of subcellular fractions of the protoplasts was next studied. A typical result, in which [^{14}C]glycine was used, is shown in Fig. 1. For periods of incubation up to about 6 min. the protein of the membrane fraction has the highest specific radioactivity, but thereafter the cytoplasmic proteins become more highly radioactive.

Table 1. Comparison of the incorporation of ^{14}C -amino acids into the proteins of cell walls and protoplasts

Labelled amino acids ($2\mu\text{C}$) were added to cells (100 mg. dry wt. at 1 mg./ml.) growing in a glucose-salts medium, and the incubation was continued as before for 5 or 30 min. The cells were then sedimented, washed and the cell walls digested with lysozyme as described in the Methods section of this paper.

Amino acid added	Specific activity of cell-wall protein ($\mu\text{C/g.}$) after		Specific activity of protoplast protein ($\mu\text{C/g.}$) after	
	5 min.	30 min.	5 min.	30 min.
L- ^{14}C Leucine	0.09	0.24	0.71	2.85
L- ^{14}C Lysine	0.37	0.39	9.8	16.5
[1- ^{14}C]Glycine	0.35	1.65	7.7	7.8
DL-[3- ^{14}C]Phenylalanine	0.14	1.3	3.5	8.5

Fig. 1. Incorporation of [1- ^{14}C]glycine into the protein of subcellular fractions of whole cells of *Bacillus megaterium*, incubated aerobically in an inorganic salts medium with glucose. The amino acid ($10\mu\text{C}$) was added to 1 g. dry wt. of cells growing at 1 mg./ml. in this medium. Samples were taken at various times and analysed as described in the Methods section. ●, Cytoplasm; ○, cytoplasmic membrane; Δ, EDTA digest.

The proteins of the EDTA digests had, in general, a lower specific radioactivity than either of the other fractions, except at the very shortest periods of incubation when they had slightly higher specific radioactivities than the cytoplasmic proteins. Similar results were obtained with other labelled amino acids, and it was usually unnecessary to separate cell-wall protein (or polypeptide material) from the cytoplasmic proteins, as most of it was

Fig. 2. Incorporation of L- ^{14}C lysine into subcellular fractions of whole cells of *Bacillus megaterium*, incubated aerobically in an inorganic salts medium with glucose. The amino acid ($12\mu\text{C}$) was added to 2 g. (dry wt.) of cells growing at 1 mg./ml. in the medium. Samples (333.3 ml. each) were taken at various times and analysed as described in the text. □, Cell wall; ●, cytoplasm; ■, non-protein from cytoplasm; ○, cytoplasmic membrane.

removed during the precipitation and washing procedures, and it did not greatly affect the observed specific activity of the cytoplasmic protein.

However, in some experiments the curves for the specific radioactivities of the different fractions were closer than in others, particularly if the lysozyme-digestion procedure was performed at a higher temperature than 2° or for a prolonged period. It seemed likely that an unknown amount of equilibration of radioactive protein between the

various fractions was taking place as long as the cells remained intact.

At the same time the rate of incorporation of L-[^{14}C]lysine into the various cellular fractions of the organism was studied as before, but the fractions were dried at 80° and the total activity present in each fraction, including the non-protein material, was determined. The results are shown in Fig. 2. A very large proportion of the added amino

acid was fixed in the cell wall and the bulk of the remainder was found in the cytoplasmic fraction. Two further points of interest were: first, the amount of label in the membrane and EDTA digests could at all times be accounted for almost entirely by the labelled protein present and, secondly, that the total radioactivity of the non-protein material present in the cytoplasm remained constant at all times of incubation from 30 sec. to

Fig. 3. Incorporation of [^{14}C]amino acids into the proteins of the membrane and cytoplasm after incubation of protoplasts of *Bacillus megaterium* in a medium containing $0.5\text{M-KH}_2\text{PO}_4$, other inorganic salts and glucose. In each case $10\text{ }\mu\text{C}$ of labelled amino acid was added to protoplasts from 1.5 g. (dry wt.) of whole cells growing at 1 mg./ml. , and samples were taken and analysed as described in the Methods section. (a) Incorporation of [1- ^{14}C]glycine; (b) incorporation of L-[^{14}C]lysine; (c) incorporation of L-[^{14}C]valine. ●, Cytoplasm; ○, cytoplasmic membrane.

20 min. These last figures were obtained by difference; after assay of the total activity, the dried cytoplasm was extracted with cold trichloroacetic acid and the radioactivity of the protein in the residue determined after the removal of lipid and nucleic acid material by standard methods.

The enormous reservoir of radioactive material within the cell walls would obviously make it difficult to interpret any experiments designed to follow the transfer of radioactive material between fractions during the course of protein synthesis. This led us to turn our attention to studies on the protoplast system, which had the twofold advantage of eliminating the cell wall and reducing the length of time required to fractionate the cellular components.

Experiments with protoplasts. In initial experiments, the great fragility of the protoplasts, combined with the necessity to maintain them under strictly aerobic conditions, made it difficult to obtain preparations that yielded consistent results. Conditions (described in the Methods section) were eventually established that gave satisfactorily reproducible results with our strain. When time-studies of the incorporation of labelled amino acids were carried out, it was found that the specific radioactivities of membrane and cytoplasmic fractions showed much greater differences than in many of the experiments carried out with whole cells. The membrane-protein fractions were again those most highly labelled at early times, but, after 5–7 min., the proteins of the cytoplasm attained higher specific radioactivities. Typical results with different ^{14}C -labelled amino acids are shown in Fig. 3. The differences between the three sets of curves probably reflect differences in the degree of damage to the protoplasts and not differences in the incorporation behaviour of the individual amino acids.

Experiments with broken protoplasts and isolated membranes. When protoplasts were disintegrated by rapid shaking in the Mickle tissue disintegrator and then incubated in the usual protoplast-incorporation medium, it was found that ^{14}C -labelled amino acids were taken up into the membrane protein at rates comparable with those observed in intact protoplasts (Fig. 4). Very little radioactivity was found in the cytoplasmic protein, however. This result was largely repeatable although in one subsequent experiment the cytoplasmic proteins reached a specific radioactivity of 25% of that of the membrane protein after incubation for 30 min.

Much the same degree of incorporation of ^{14}C -labelled amino acids into membrane proteins was found if the membranes were separated from the cytoplasm by sedimentation and resuspended in 'C' solution with 1% added glucose before carrying out the incubation (Fig. 5). The presence of traces

of cytoplasm was not necessary because membranes twice washed with buffer were as active as membranes used immediately after sedimentation. The method used for breaking the protoplasts was also unimportant, as it was found that membranes prepared from protoplasts lysed by diluting the medium three times with water incorporated labelled amino acids into protein just as well as those obtained by breaking protoplasts in the Mickle disintegrator. Finally, full activity of the system was retained if the protoplasts were lysed and the membranes collected and used after storage for 2 days at 2° (Table 2).

The linear incorporation of labelled amino acids into membrane protein was found to continue for at least 2 hr. with L- ^{14}C valine (Fig. 5). In other experiments, incubations were not continued for such a long period but the uptake was linear until the end of the experiment in all.

Fig. 4. Incorporation of $[1-^{14}\text{C}]$ glycine into the protein of the membrane fraction of disrupted protoplasts suspended at a density of 1 mg./ml. in a medium containing 0.5 M- KH_2PO_4 , other inorganic salts and glucose. The amino acid ($10\text{ }\mu\text{Ci}$) was added to membranes derived from 1.5 g. (dry wt.) of whole cells. The disruption and subsequent analysis are described in the Methods section.

Table 2. Incorporation of $[1-^{14}\text{C}]$ glycine into membranes prepared from lysed protoplasts stored at 2° for varying lengths of time

Incorporation of $2\text{ }\mu\text{Ci}$ of labelled amino acid was carried out for 30 min.; 100 mg. of membranes was used in each case. Other experimental conditions were as described in the text.

Time of storage of lysed protoplasts (days)	Incorporation of labelled amino acids into membrane protein ($\mu\text{Ci/g.}$)
0	8
1	11
2	7

The action of deoxyribonuclease on the system was also examined. Membranes were incubated with the enzyme before and after incubation with ¹⁴C-labelled amino acid and the results are summarized in Table 3. These treatments had little effect although in both a small amount of protein was solubilized. However, prolonged incubation of

the membranes at 37° with or without deoxyribonuclease did considerably reduce the rate at which amino acids were incorporated into the protein of the membranes.

Transfer of labelled protein from membranes to cytoplasm. At the same time, repeated attempts were made to obtain incorporation of ¹⁴C-labelled amino acids into the proteins of isolated cytoplasm, diluted by the medium in which the protoplasts were broken. A small incorporation was observed, never more than 10 % of the incorporation into isolated membranes and usually much less. The incorporation was not enhanced by including adenosine triphosphate, fructose 1:6-diphosphate or a complete mixture of amino acids in the medium. However, it was found that a very high degree of labelling of the cytoplasmic proteins could be achieved if they were incubated with washed membranes previously labelled *in vitro* as described above. Further, the proportion of radioactive label that could be transferred from membrane to cytoplasm was dependent upon the time for which the membranes had been incubated with the [¹⁴C]amino acid. The results are summarized in Table 4, and it will be seen that no loss of labelled

Fig. 5. Incorporation of L-[¹⁴C]valine into the proteins of the membrane fraction isolated from disrupted protoplasts of *Bacillus megaterium*. The amino acid (10 μC) was added to membranes derived from 1.5 g. of whole cells suspended at 1 mg./ml. in a medium containing inorganic salts and glucose. The preparation of the fraction and subsequent analysis of the samples taken at various times is described in the Methods section.

Table 3. *Effect of digestion with deoxyribonuclease on the specific radioactivity of the membrane proteins*

Membranes (100 mg.) were treated at 37° for 1 hr. with deoxyribonuclease (DNA-ase; 50 μg./ml.) in 10 ml. of 'C' solution-phosphate medium.

The membranes were then sedimented and either resuspended for incubation or treated with trichloroacetic acid. Incubations were carried out with 2 μC of [1-¹⁴C]-glycine as described in the text.

Treatment with DNA-ase before incubating membranes for 2 min. with labelled amino acid		Treatment with DNA-ase after incubating membranes for 30 min. with labelled amino acid	
DNA-ase	Specific activity of protein (μC/g.)	DNA-ase	Specific activity of protein (μC/g.)
+	0.36	+	3.9
-	0.38	-	4.1

Table 4. *Transfer of a labelled protein fraction from membranes labelled in vitro with [1-¹⁴C]glycine to the protein fraction of unlabelled cytoplasm*

In each case, the membranes (approx. dry wt. 42 mg.) were incubated with cytoplasm (approx. dry wt. 93 mg.) for 5 min. All incubations were carried out aerobically at 30°.

Time of incubation of membranes with [1- ¹⁴ C]glycine (min.)	Initial total radioactivity of membrane proteins (μmc)	Radioactivity of membrane proteins after incubation with cytoplasm (μmc)	Final radioactivity of cytoplasmic proteins (μmc)	Transfer (%)
1.5	2.7	1.1	1.6	59
10	22	15	8	36
30	51	39	10	20

material from the combined protein fractions occurs, and that the greatest percentage transfer was obtained with membranes that had been allowed to incorporate for the shortest time.

An attempt was made to determine the rate at which labelled protein is transferred from the membrane to the cytoplasm, membranes which had been pre-incubated for 20 min. with [14 C]-amino acid being used. It was concluded that this is a very rapid process as the transfer was found to be complete in 1 min., the smallest interval measured. On the other hand, very little radioactivity (never more than 5%) was lost from the protein fraction of previously labelled membranes when they were incubated in buffer alone for 30 min.

Analyses. The nucleic acid contents of whole cells, membranes and cytoplasm as determined by the methods of Cerriotti (1952, 1955) are given in Table 5. The values obtained differ from those given by Weibull (1953b), but his preparative procedures differed in that he prepared protoplasts in a sucrose medium and, probably more important, subsequently treated them with deoxyribonuclease before examining membranes and cytoplasm.

Crude nucleic acid isolated from the cytoplasm directly by precipitation of the proteins with sodium dodecyl sulphate was found by base analysis to consist of 89% of RNA and 11% of DNA, in good agreement with the results given by the colour reactions. But the treatment of finely divided membranes with sodium dodecyl sulphate liberated nucleic acid into solution that was shown, after purification, to contain only traces of RNA. Weibull's membranes contained very little RNA, but the bulk of the RNA could be extracted from the cytoplasm by centrifuging for 2½ hr. at 105 000 g and sedimentation studies showed that there were three macromolecular components present with sedimentation coefficients of 42, 26 and 3s. In our preparations sedimentation analysis of the cytoplasm, performed by Dr K. V. Shooter of this Institute, showed that there were three macromolecular components with sedimentation coefficients of 13, 6 and 3s and at least 80% of the material was present in the 3s component.

The cytoplasm, as prepared by us, thus contains no particles comparable with microsomes. In fact it can be estimated that the 3s component has a molecular weight of about 30 000.

DISCUSSION

The initial experiments with whole cells show that although the cell wall contains large quantities of free amino acids, it plays very little part in, at least, the initial processes of protein synthesis in the remainder of the cell. It may act as a reservoir of amino acids and provide a constant supply to the organism. This could account for the consistency of the amount of unincorporated amino acids found in the cytoplasm which are either in the free state or in the form of simple derivatives.

In the whole-cell experiments the time required for the digestion by lysozyme probably did result in some equilibration between the labelled protein of the membrane and cytoplasmic fractions, because, in the more rapidly fractionated protoplast experiments, the differences in the labelling of these fractions are much more marked. Although all experiments show an initially greater labelling of the membrane, which is later exceeded by the cytoplasm, individual differences observed in experiments are difficult to interpret. It is, however, not easy to be certain that, in any one experiment, all the protoplasts are active or even that all of them are intact.

However, the feature common to all these curves strongly suggests that the initial incorporation of amino acid into protein occurs in the membrane fraction and is followed by transfer to the cytoplasm. The further rise, usually observed after 6 min., in the specific activity of the proteins of the cytoplasmic membranes is then explicable if protein, initially formed in the membranes, can be used, possibly after further modification for structural or enzymic purposes in the membranes themselves as well as in the cytoplasm.

Our conclusions are supported by the results of the experiments in which the isolated membranes were labelled *in vitro*. It is to be noted that, in many cases, a similar level of labelling was observed with isolated membranes as with membranes from whole protoplasts. That the cytoplasm does not play a vital part in this process is borne out by the fact that its complete removal by washing did not prevent incorporation. A suggestion that the cytoplasmic membranes of *B. megaterium* may be a site of protein synthesis has also been made elsewhere (McQuillen, 1956).

The actual amount of amino acid incorporated into the proteins is sufficient to show that the mechanism studied cannot be dismissed as an insignificant process. Thus in one experiment an

Table 5. *Composition of whole cells, cytoplasmic membranes and cytoplasm of Bacillus megaterium, prepared as described in the text*

	Dry wt. of whole cells (%)	DNA (%)	RNA (%)
Whole cells	100	1.5	8.0
Cytoplasmic membranes	29	2.8	3.7
Cytoplasm	43	1.3	9.9

incorporation of 50 μ g. of [1- 14 C]glycine into 50 mg. of membrane proteins was observed; the labelling was not reduced by dialysing the membranes against four changes of water at 1° before adding trichloroacetic acid. This amount represents an incorporation equivalent to about 2% of the pre-existing glycine residues.

The length of time for which the membranes will continue to incorporate 14 C-labelled amino acids into their protein fraction is also remarkable, the system being much more active in this respect than other systems *in vitro* already described. The part played by the cytoplasm in the intact cell is not clear. It appears to be the final repository of the bulk of the label and thus may be the eventual receptacle of many newly formed proteins. The transfer of labelled protein from membranes to cytoplasm removed previously is a very rapid process and occurs without loss of labelled protein. This suggests that protein is being transferred rather than amino acids or simple derivatives and is in itself indirect confirmation that the processes studied *in vitro* are closely allied to protein synthesis.

Much less radioactivity appeared in the cytoplasmic-protein fraction in the broken-protoplast experiments than in the transfer experiments. The reason for this discrepancy is not clear but could be explained by production of a soluble transfer inhibitor in the cytoplasmic membranes which is excreted into the medium in the whole-protoplast experiments and is also kept from contact with the cytoplasm by an intervening sedimentation in the transfer experiments.

We can conclude that these experiments give an indication that the main site of protein synthesis in *B. megaterium* is the cytoplasmic membrane. It must be remembered that the 'membrane fraction' obtained in these experiments includes not only the actual protoplast membrane, but also the lipid granules and part, at any rate, of the DNA gel. However, the cytoplasmic membranes themselves constitute the bulk (75%) of the fraction, and there seems little doubt that they provide the sites for the incorporation of labelled amino acids. Thus our experiments with deoxyribonuclease make it seem unlikely that DNA or protein associated with it plays an important role in the incorporation process. This is further borne out by the experiments with EDTA which showed, although the results obtained were rather variable, that protein solubilized with the bulk of the DNA, and possibly associated with it, was not as readily labelled as the residual protein. Besides, a large proportion of the cellular DNA occurs in the cytoplasmic fraction, and the material of the original DNA-containing gel is probably distributed almost equally between our membrane and cytoplasmic fractions.

It seems most unlikely that the lipid granules play any direct part in protein synthesis: these also are digested by EDTA. The 'small-particle' fraction obtained by Weibull (1953*b*) is absent from our cytoplasm. Whether this arises from the dissolution of the gel by Weibull's deoxyribonuclease or the cytoplasmic membranes is an open question; but Mitchell & Moyle (1956) have shown that the 'small-particle' fraction of *Staphylococcus aureus* probably arises as a result of the disintegration of the delicate cytoplasmic membrane of that organism. Methods of fractionating the cytoplasmic membranes are at present being studied in more detail.

SUMMARY

1. The incorporation of 14 C-labelled amino acids into protein has been studied in whole cells of *Bacillus megaterium* and also in protoplasts and various subcellular fractions derived from the same organism.

2. The cell wall does not incorporate any of the amino acids studied into its 'protein' to any great extent, but it absorbs a large quantity of free amino acid.

3. Initially, labelled amino acids are incorporated most rapidly into the protein derived from the cytoplasmic membranes of whole cells and protoplasts incubated in growth media. At later times the cytoplasmic proteins attain higher specific activities. At all times the level of the non-protein radioactivity is highest in the cytoplasm.

4. Isolated cytoplasmic membranes can incorporate 14 C-labelled amino acids into their protein fractions at rates comparable with those observed in the membranes of whole protoplasts: the incorporation is linear for at least 2 hr. In comparison, isolated cytoplasm incorporated very small amounts of amino acid into protein.

5. If membranes labelled in this way are incubated with non-labelled cytoplasm, a considerable proportion of the radioactivity is rapidly transferred from the membrane protein to the cytoplasmic protein.

6. It is concluded that the initial stages of protein synthesis in *B. megaterium* take place at sites on or closely associated with the cytoplasmic membrane.

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The Metabolic Stability of the Nucleic Acids in Cultures of a Pure Strain of Mammalian Cells

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Although in recent years the metabolism of the nucleic acids has been the subject of many experiments with isotopic-tracer techniques, our knowledge of the relative rates at which deoxyribonucleic acid (DNA), nuclear ribonucleic acid (nRNA) and cytoplasmic ribonucleic acid (cRNA) are synthesized and broken down is still far from complete.

The present experiments were designed to provide answers to two particular questions: (1) What inferences about nucleic acid synthesis can be drawn from the manner in which [¹⁴C]formate is incorporated into the purines and pyrimidines of DNA, nRNA and cRNA? (2) To what extent are DNA, nRNA and cRNA, once synthesized, metabolically stable in animal cells?

Information of this kind cannot readily be obtained from the intact animal or from tissue slices surviving *in vitro*. Use was therefore made of a pure strain of mammalian cells growing in tissue culture. Brief preliminary reports of some of the results obtained have already been published (Thomson, Paul & Davidson, 1956; Thomson & Paul, 1957).

METHODS

Biological

The cells used in these experiments were adult mouse subcutaneous fibroblasts NCTC strain L, clone 929 (Sanford, Earle & Likely, 1948), hereafter referred to as L cells. These

cells were grown directly on the floor of large conical culture flasks having an area of about 140 cm.². The medium in which they were normally maintained was composed of 20 parts of horse serum, 10 parts of chick-embryo extract and 70 parts of Hanks's balanced salt solution (Hanks & Wallace, 1949). It also contained 50 units of sodium penicillin G/ml. but no other antibiotics.

In most of the experiments to be described, similar vessels were used. In each vessel was placed a glass-covered magnet so that the cells could readily be suspended by placing the culture vessel on a magnetic stirrer. This procedure proved less damaging to the cells than treatment with trypsin. In the conditions described the cells usually reached a maximum population of between 50 and 80 millions/flask, at which level the population became stationary. In all experiments the population was kept near the maximum, the cultures being halved at each transfer and allowed to grow to the maximum once more. The medium, 60 ml./flask, was completely renewed twice a week and on one of these occasions the cells were transferred.

In this way, a slow rate of growth was maintained and the cells remained healthy throughout. In some experiments, where it was necessary to exclude extracellular pools of purines and pyrimidines, the medium used was Eagle's medium (Eagle, 1955) supplemented with 30% (v/v) of dialysed horse serum. Also, in some experiments where the inocula of cells were much smaller than those described above, smaller vessels were used, e.g. 25 ml. Erlenmeyer flasks.

At the commencement of an experiment a suspension of cells was obtained by treating stock cultures of cells with