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INCORPORATION OF [^{14}C]GLYCINE INTO *MICROCOCCUS LYSODEIKTICUS* MEMBRANE PROTEIN AND EFFECTS OF PROTEIN SYNTHESIS INHIBITORS

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SUMMARY

[^{14}C]Glycine incorporation into *Micrococcus lysodeikticus* membranes and membrane protein showed a variable level of resistance to the inhibitory effects of chloramphenicol at 100 μg/ml, a concentration which inhibited the incorporation into the cytoplasmic protein fraction to the extent of 90–97 %. Incorporation into the membrane protein in the presence of chloramphenicol ranged from 30 to 100 % that observed for control (no chloramphenicol) membrane protein fractions. Only 1 % of the total incorporation, both in the presence and absence of chloramphenicol, could be accounted for as N-terminal groups of the proteins. Over 80 % of the incorporated [^{14}C]glycine was recovered as glycine after acid hydrolysis and on high-voltage electrophoresis of pronase digests, the label migrated as free glycine and peptides. Fractionation of the membrane proteins after lipid extraction with *n*-butanol revealed that the "water-soluble protein" fraction was the least affected by chloramphenicol and that incorporation into the "water-insoluble protein" fraction was inhibited to the extent of 75 %. No such differential inhibitory effect upon incorporation into cytoplasmic and membrane proteins was observed with puromycin (5 μg/ml) which inhibited the uptake into both cellular fractions to an equal extent (80 % after 90 min preincubation with the inhibitor). Cycloheximide was ineffective on both growth and incorporation of [^{14}C]glycine.

INTRODUCTION

The biogenesis of cell membranes and membranous organelles such as mitochondria and chloroplasts has attracted a great deal of attention in recent years (*e.g.* refs. 1–3). There is now a large body of information on the incorporation of radioactive substances into membrane proteins and lipids (*e.g.* refs. 4, 5) and some of these studies have given an idea of the degrees of interdependence of the biosynthesis of the proteins and lipids[6–8]. A much clearer understanding of the biosynthesis of mitochondria has been gained during the past few years from studies of the inhibitory effects of antibacterial agents. Thus the synthesis of the yeast mitochondrial inner membrane and its cytochromes *a*, a_3, *b* and c_1 is inhibited by a number of antibiotics including chloramphenicol[2,9]. The antibiotics did not, however, interfere with the

formation of the outer membrane or certain soluble enzymes of yeast mitochondria[9]. This has led to the conclusion that the synthesis of these inner mitochondrial membrane components is catalysed by 70-S ribosomes and that the formation of the outer membrane is mediated by the 80-S, "cytoplasmic" ribosomes. Similar mechanisms appear to be involved in both animal[10] and yeast mitochondria and chloroplast membranes[11,12]. The existence of 70-S ribosomes in chloroplasts was first established by BOARDMAN *et al.*[13].

Although bacteria do not possess a completely separate mitochondrial organelle, it is evident that the mitochondrial functions for electron transport and oxidative phosphorylation reside in their membrane systems[14]. Apart from the studies of WHITE[15,16] and the recent investigation of KAHANE AND RAZIN[8], there is very little information on the formation and biogenesis of bacterial membranes and their respiratory components. In order to gain some insight into the biosynthesis of a bacterial membrane, we selected *Micrococcus lysodeikticus* which has a well-developed membrane system and is a strict aerobe possessing a powerful system for oxidative phosphorylation. The results of our investigations on the incorporation of [^{14}C]glycine into *M. lysodeikticus* membrane proteins are presented in this paper together with the influence of chloramphenicol and other inhibitors on membrane and cytoplasmic protein synthesis.

MATERIALS AND METHODS

Growth conditions and membrane isolation

M. lysodeikticus (NCTC 2665) was grown in the peptone–water–yeast extract medium as previously described[17] and aerated by vigorous shaking in a New Brunswick incubator shaker at 30°. Overnight inocula were used for each experiment and these were added to sterile, diluted (1:8) peptone–water–yeast extract medium. Cultures of diluted peptone–water–yeast extract medium containing a 5% (by volume) inoculum of the overnight culture were allowed to undergo about a doubling of the absorbance before the radioactive amino acid and/or the inhibitors were added (Fig. 1).

To arrest the growth and label incorporation, culture samples (20–50 ml) taken at various intervals of time were added to 100 ml of ice-cold diluted peptone–water–yeast extract medium. Bacteria were harvested by centrifugation in the cold and

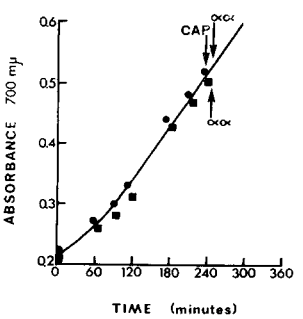

Fig. 1. Growth of *M. lysodeikticus* on diluted (1:8) peptone–water–yeast extract medium at 30°, indicating the stages at which chloramphenicol (CAP) and [^{14}C]glycine (ααα) were added to the culture.

each sample pellet was washed once with 100 ml of cold, dilute peptone–water–yeast extract medium. The small residual volumes of the wash media were carefully removed from the cell pellets with pasteur pipettes. Each pellet was then suspended in 7 ml of cold, 25 mM Tris–HCl buffer (pH 7.5) transferred to stainless steel Spinco centrifuge tubes and a solution containing 500 μg lysozyme (EC 3.2.1.17) (Worthington Biochemical Corp. crystalline, egg-white lysozyme) was added to each of the cell suspensions at 0° and immediately incubated at 37°. Bacterial lysis was rapid and after 10 min, deoxyribonuclease (240 μg deoxyribonuclease/7-ml sample) was added and the tubes held at 37° for an additional 10 min. Membranes were then recovered from the lysate by centrifugation at 67000 $\times$ g for 30 min at 0–5°. The supernatant, constituting the "cytoplasmic fraction", was poured off and the membrane pellets were washed once, in the cold, with 5 ml 25 mM Tris buffer (pH 7.5), finally suspended in 1 or 2 ml Tris buffer (25 mM, pH 7.5) and designated as the "membrane fraction". More concentrated cytoplasmic fractions had to be diluted with buffer and recentrifuged at 67000 $\times$ g to remove residual membrane fragments.

Chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) was usually added to the growing culture 5 min before the addition of the labeled amino acid, to give a final antibiotic concentration of 100 μg/ml in all experiments. Uniformly-labeled [^{14}C]-glycine (> 60 mC/mmole) from New England Nuclear Corp., Boston, Mass. was used throughout these studies. For most of the experiments [^{14}C]glycine was added to give 0.1–0.5 μC/ml culture of *M. lysodeikticus* in the diluted peptone–water–yeast extract medium.

Preparation of membrane and cytoplasmic protein fractions

[^{14}C]Glycine incorporation into membrane and cytoplasmic protein fractions was determined as follows: membrane preparations and cytoplasmic fractions were treated with trichloroacetic acid (10% (w/v) final concentration) for 15–30 min at 0° and the precipitates washed with 5% trichloroacetic acid. The precipitates were collected by centrifugation and the trichloroacetic acid-insoluble residues were extracted with 5 ml acetone–methanol (7:2, v/v) for 1 h at 37° as previously described[18] to remove the lipids. The solvent was pipetted off after centrifugation and the pellet was air-dried for a few minutes and the protein residues were dissolved or dispersed in 0.2–0.4 ml 1 M NaOH (not all of the membrane protein dissolved). After 20–30 min, NaOH was neutralized with HCl and the proteins were again precipitated with 10% trichloroacetic acid and the residues dissolved in 0.2–0.5 ml 0.5 M NaOH. Membrane samples were homogenized with 0.5 M NaOH containing 0.5% (w/v) sodium dodecyl sulfate and these samples were used for determinations of protein contents and radioactivity.

In some experiments, membrane protein fractions were obtained in an aqueous phase after removal of the lipids by *n*-butanol extraction. Membranes suspended in 25 mM Tris (pH 7.5) were homogenized with an equal volume of ice-cold, *n*-butanol and allowed to stand in an ice-bath for 15 min. The preparations were then centrifuged at 30000 $\times$ g for 20 min to break the emulsion and the upper solvent phase containing lipids and carotenoid pigments was removed and the interfacial layer containing insoluble proteins was rinsed several times with ice-cold *n*-butanol. The butanol dissolved in the aqueous phase was removed by dialysis against either 25 mM Tris buffer (pH 7.5) or distilled water. This aqueous phase contained insoluble proteins

in suspension and they could be separated from the soluble protein fraction by low-speed centrifugation (5000–10000 × *g*, for 15 min).

Proteins were determined by the method of LOWRY *et al.*[19], using bovine serum albumin as a standard. Incorporation of [^{14}C]glycine into the N-termini of the membrane proteins was determined by reacting the labeled membranes with FDNB as described by GHUYSEN *et al.*[20], after prior trichloroacetic acid precipitation and acetone–methanol extraction of the membrane fractions. The ether-soluble DNP-amino acid fraction was obtained after acid hydrolysis of the reacted membranes by the method of GHUYSEN *et al.*[20].

Radioactivity measurements were performed by applying 0.01–0.09-ml samples of membranes, proteins or lipid fractions to Whatman No. 3MM paper discs, allowing them to dry and then placing the discs in vials containing 5 ml counting fluid (42 ml Liquifluor, product of Nuclear-Chicago, mixed with 1 l toluene). The samples were counted in a Nuclear-Chicago liquid scintillation counter, model Mark I.

RESULTS

Incorporation of [^{14}C]glycine into membrane and cytoplasmic fractions

The incorporation of [^{14}C]glycine into the cytoplasmic and membrane fractions of *M. lysodeikticus* in the presence and absence of chloramphenicol (100 μg/ml) is illustrated by the results of a typical experiment in Fig. 2. In accordance with earlier studies[21], chloramphenicol rapidly inhibited the uptake of [^{14}C]glycine into the cytoplasmic protein fraction and the level of inhibition was 90–97%. However, as shown in Fig. 2 only partial inhibition of [^{14}C]glycine incorporation into membrane protein by chloramphenicol was observed and over a series of many experiments the inhibitory level has ranged from 0 to 70%. It has also been repeatedly noted (as in Fig. 2) that [^{14}C]glycine appeared in the membrane protein somewhat more rapidly during the first few minutes than it did in the cytoplasmic protein. The incorporation of [^{14}C]-

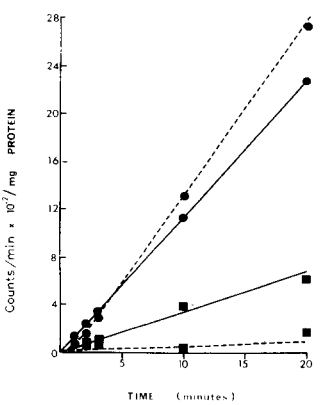

Fig. 2. The course of incorporation of [^{14}C]glycine into membrane and cytoplasmic protein fractions from *M. lysodeikticus* grown at 30° was determined at intervals for 20 min in the presence and absence of chloramphenicol at a level of 100 μg/ml. Figure shows [^{14}C]glycine incorporation into the cytoplasmic protein fractions from control (no chloramphenicol), ●----●; and chloramphenicol-treated cells, ■----■; and the corresponding membrane fractions: ●——●, no chloramphenicol; ■——■, + chloramphenicol.

glycine into the membrane protein fraction in the presence of chloramphenicol was, moreover, linear for at least 60 min as shown in Fig. 3.

Some label from [^{14}C]glycine also found its way into the membrane lipid fraction as illustrated in Fig. 4. Although initially this incorporation was rapid, after about

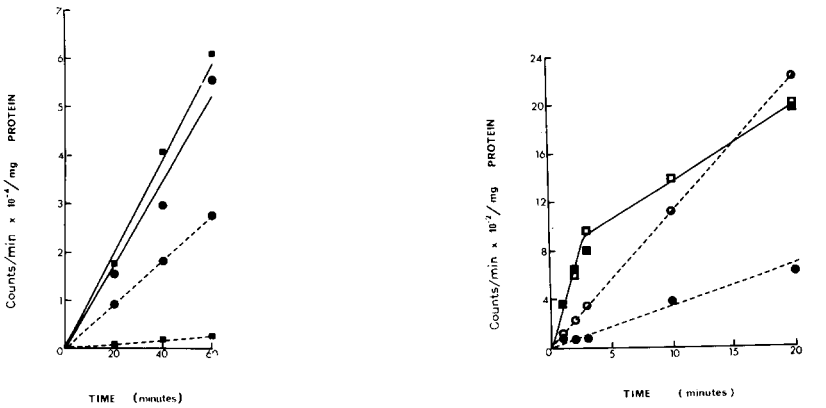

Fig. 3. The incorporation of [^{14}C]glycine into *M. lysodeikticus* membrane and cytoplasmic fractions was determined at 20-min intervals for up to 60 min of growth in the presence and absence of chloramphenicol at 100 μg/ml. Membrane protein control (no chloramphenicol), ●———●; membrane protein + chloramphenicol, ■———■; cytoplasmic protein control (no chloramphenicol), ●----●; cytoplasmic protein + chloramphenicol, ■----■.

Fig. 4. [^{14}C]Glycine incorporation into membrane lipid and protein fractions of *M. lysodeikticus* grown at 30° in the presence and absence of chloramphenicol at 100 μg/ml. Lipid fraction (no chloramphenicol), □———□; lipid fraction + chloramphenicol, ■———■; membrane protein (no chloramphenicol), ○----○; membrane protein + chloramphenicol, ●----●.

3 min it began to level off and upon longer periods of incubation (*e.g.* 40 min) it seldom exceeded 10% of the counts found in the membrane fractions. Chloramphenicol was without effect on the incorporation into the membrane lipid fraction. Although the nature of the material labeled by [^{14}C]glycine and extracted by organic solvents has not been investigated, the lack of linearity in the appearance of label in the lipid fraction would argue against simple breakdown of the [^{14}C]glycine and reutilization of its carbon for lipid synthesis.

Further attempts were made to determine whether or not the [^{14}C]glycine had become an integral part of the membrane protein fraction. Although it seemed unlikely that bound [^{14}C]glycine would be retained in the protein fractions prepared as described in MATERIALS AND METHODS, the membranes were subjected to several additional extraction procedures. Lipid-extracted [^{14}C]glycine-labeled membranes were either heated with 5% trichloroacetic acid, treated for 20 min with 1 M NaOH (as described in MATERIALS AND METHODS) or treated for 90 min with 1 M NaOH. Subjecting the membrane protein fractions to these treatments did not significantly alter the incorporation pattern as summarized in Table I. The fact that prolonged treatment with alkali and extraction with trichloroacetic acid did not lead to significant lowering of the radioactivity in the precipitated protein fractions minimizes the possibility that the incorporation could have been due largely to the presence of ribosomes carrying incomplete or nascent peptide chains. Such procedures as those

TABLE I

EFFECTS OF SUBJECTING LABELED MEMBRANE PROTEIN FRACTIONS OF *M. lysodeikticus* FROM UNTREATED (NORMAL) AND CHLORAMPHENICOL-TREATED CELLS TO FURTHER EXTRACTION TREATMENTS

Treatment	*Membrane*	[^{14}C *Glycine incorporated (counts/min per mg protein)*
20 min 37°, 1.0 M NaOH	Normal	108 000
	Chloramphenicol	136 000
90 min 37°, 1.0 M NaOH	Normal	104 000
	Chloramphenicol	146 000
15 min 100°, 5% trichloroacetic acid	Normal	106 000
	Chloramphenicol	103 000

TABLE II

INCORPORATION OF [^{14}C]GLYCINE INTO N-TERMINAL AMINO ACID FRACTION OF MEMBRANE PROTEINS IN PRESENCE AND ABSENCE OF CHLORAMPHENICOL

^{14}C Glycine-labeled membranes were trichloroacetic acid-precipitated and acetone–methanol-extracted as described in MATERIALS AND METHODS. N-terminal amino acid analysis was performed on dried, extracted membranes. The specific activity of these membrane proteins was approx. 100000 counts/min per mg protein. Each sample used for N-terminal amino acid determination by reaction with FDNB contained approx. 74000 counts/min. After hydrolysis, counts were determined on the ether-soluble (DNP-amino acids) phase and the acid-soluble fraction (free amino acids).

	DNP-amino acids (ether phase)	*Free amino acids (acid-soluble phase)*	*% of N-terminal* [^{14}C]*glycine*
Membrane, control	770	67 725	1.1
Membrane, chloramphenicol	525	52 745	1.0

used in this study have also been used by other investigators to reduce ribosomal contamination (*e.g.* refs. 4, 5, 28, 34–36). Moreover, under our conditions of preparing the membranes in the absence of Mg^{2+}, RNA accounts for less than 1% of the membrane structure.

The possibility that the incorporation observed was due simply to the addition of [^{14}C]glycine to the N-terminal ends of peptide chains was explored since several systems catalysing N-terminal additions of amino acids to proteins have been reported[22,23]. The data presented in Table II show that only about 1% of the [^{14}C]glycine incorporated appeared as N-terminal end groups of the proteins and that chloramphenicol did not alter this level of incorporation into N-termini.

Evidence that the [^{14}C]glycine incorporation is into peptide in the membrane protein fractions was further sought by examining the products of pronase digestion of [^{14}C]glycine-labeled membranes. Pronase digests of both control membranes and membrane fractions labeled in the presence of chloramphenicol were examined for radioactivity after high-voltage electrophoresis and the pattern of labeling in Fig. 5 suggests the presence of an equal number of bands containing [^{14}C]glycine. Undigested [^{14}C]glycine-labeled membranes showed no migration from the point of origin. Pronase digests of trichloroacetic acid-precipitated, lipid-extracted, and

NaOH-treated samples from the same membrane fractions behaved similarly on high-voltage electrophoresis. In each case, however, pronase liberated more free glycine from the membrane sample which had been labeled in the presence of chloramphenicol. It is thus conceivable that membrane proteins which are synthesized in the

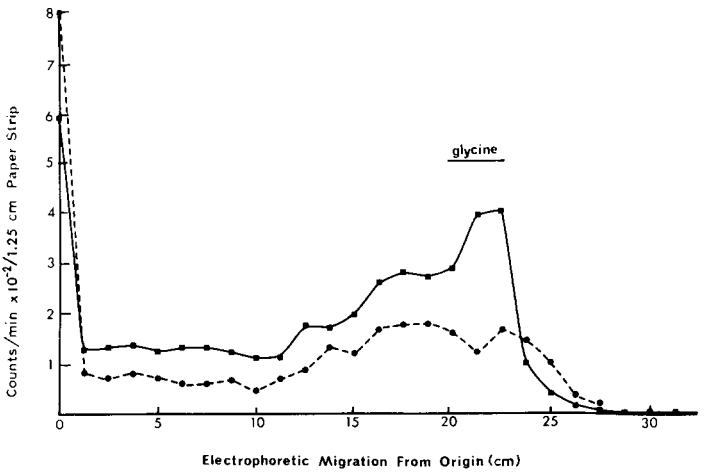

Fig. 5. High-voltage electrophoresis pattern of [14C]glycine-labeled, pronase-digested membranes of *M. lysodeikticus* grown in the presence (■——■) and absence (●--●) of chloramphenicol at 100 μg/ml. Approx. 500 μg of each of the control and chloramphenicol-treated, [14C]glycine-labeled washed whole membranes were digested with 100 μg pronase for 16 h at 37° in a final volume of 0.45 ml (50 mM Tris–HCl buffer, pH 7.5). Samples of digested and undigested membranes were applied on Whatman paper No. 3MM and electrophoresed for 3 h at pH 1.9 (buffer and conditions of CEDAR AND SCHWARTZ[37]). Electrophoresis papers were cut into strips (1.25 cm) and radioactivity counted in the liquid scintillation counter. None of the radioactivity in the undigested samples (not shown in figure) migrated beyond the origin. The location of free glycine under identical conditions of electrophoresis is indicated above the curves for digested membranes.

presence of chloramphenicol are richer in glycine. Hydrolysis (4 M HCl, sealed ampoules at 110° for 20 h) of the pronase digests of membranes (Fig. 5) or lipid-extracted membranes, revealed on two-dimensional paper chromatography in the system previously described[24] that in all cases 82–89% of the radioactivity was recovered in the glycine spots on the chromatograms.

Differential sensitivity of membrane protein fractions to inhibitory effects of chloramphenicol

The variability in the inhibitory effects of chloramphenicol on [14C]glycine incorporation into the total protein fraction of *M. lysodeikticus* membranes has been noted above and at the present time we have no obvious explanation of this feature of our observations. It is conceivable that not all membrane proteins are being synthesized simultaneously and that these variable effects could be due to differences in the variety of the specific proteins being synthesized at any given time. Such a situation could account for the lack of chloramphenicol inhibition observed in some of our experiments. Studies with *M. lysodeikticus* membranes in this laboratory have established that the relative strengths of association (probably of a hydrophobic nature) between membrane proteins and lipids show considerable specificity[25]. The least soluble proteins of the membrane constitute the cytochromes *a*, *b*, *c* and succinate

dehydrogenase complement of *M. lysodeikticus* membranes[26]. It was therefore of interest to determine whether chloramphenicol partially depressed all of the membrane proteins or if it specifically inhibited the synthesis of only a certain fraction of the proteins.

TABLE III

EFFECTS OF CHLORAMPHENICOL (100 μg/ml) ON [^{14}C]GLYCINE INCORPORATION INTO *M. lysodeikticus* CYTOPLASMIC AND MEMBRANE PROTEIN FRACTIONS

Membrane suspensions were extracted with *n*-butanol to remove lipids as described in MATERIALS AND METHODS and the aqueous phases separated into water-soluble protein and water-insoluble protein fractions.

Fraction	*Specific activity (counts/min per mg protein)*	*Inhibition (%)*
Cytoplasmic protein, control	14 500	
Cytoplasmic protein, + chloramphenicol	414	97
Membrane, control	31 200	
Membrane, + chloramphenicol	19 000	39
Membrane water-soluble protein, control	44 600	
Membrane water-soluble protein, + chloramphenicol	53 200	−19
Membrane water-insoluble protein, control	24 000	
Membrane water-insoluble protein, + chloramphenicol	6 100	75

Growing cells of *M. lysodeikticus* were accordingly exposed to [^{14}C]glycine for 20 min in the presence and absence of chloramphenicol. Lysates from the two cultures were fractionated and assayed for [^{14}C]glycine incorporation and the results are presented in the upper section of Table III. The corresponding membrane fractions from the control and chloramphenicol-treated cultures were further fractionated by extracting lipids with cold *n*-butanol as described in MATERIALS AND METHODS. From the data summarized in the lower part of Table III, it appears that chloramphenicol specifically inhibited the incorporation of [^{14}C]glycine into the water-insoluble membrane proteins, *i.e.* the fraction containing the cytochromes and succinic dehydrogenase activity. Furthermore, the specific activity of the control membrane and water-insoluble fraction from *n*-butanol-extracted membranes approached that of the specific activity of the corresponding cytoplasmic protein fractions. Chloramphenicol had no inhibitory effect on [^{14}C]glycine incorporation into the soluble membrane protein fraction (*i.e.* soluble, aqueous-phase fraction after *n*-butanol extraction). Indeed, as shown in Table III, the specific activity of this latter fraction was about 20% higher in the presence of chloramphenicol.

Influence of other inhibitors of protein synthesis

Puromycin inhibits protein synthesis catalysed by both 70- and 80-S ribosomal systems[27]. The growth of *M. lysodeikticus* cultures was completely inhibited, as determined by absorbance measurements, by 5 μg puromycin/ml diluted peptone–water–yeast extract medium after 160–220 min at 30° (*i.e.* approximately one mean generation time). The effect of puromycin on the incorporation of [^{14}C]glycine into both the cytoplasmic and membrane protein fractions was tested and the results are sum-

TABLE IV

PUROMYCIN INHIBITION OF [14C]GLYCINE INCORPORATION INTO CYTOPLASMIC AND MEMBRANE PROTEIN OF *M. lysodeikticus*

Puromycin was added to a final concentration of 5 μg/ml. After 20 min in Expt. I and 90 min in Expt. II, [14C]glycine was added and the cultures were incubated for an additional 30 min. [14C]Glycine incorporation into protein was determined as described in MATERIALS AND METHODS. The growth of similar cultures was completely inhibited by 5 μg puromycin/ml after 160–220 min.

Expt. No.	*Preincubation period (min)*	*Incorporation of [14C]glycine into*		*Inhibition (%)*	
		Cytoplasmic protein (counts/min per mg protein)	*Membrane protein (counts/min per mg protein)*	*Cytoplasmic protein fraction*	*Membrane protein fraction*
I	20	9320	20 420		
	20 + puromycin	2420	8 700	71	58
II	90	8100	26 300		
	90 + puromycin	1500	5 300	80	80

marized in Table IV. The rate of inhibition of [14C]glycine entry into protein by puromycin was slower for the membrane than that observed for the cytoplasmic proteins but as shown in Table IV, upon longer preincubation with puromycin, the inhibitory effects on incorporation into membrane and cytoplasmic proteins became similar.

One other feature worthy of note in this (Table IV) and other experiments with *M. lysodeikticus*, is the higher specific activity of the membrane protein compared to that of the cytoplasmic protein fractions. Specific activities of the membrane proteins were often 2 or 3 times greater. It will be recalled that a few years ago TANI AND HENDLER[28] found that membrane fractions from *Escherichia coli* spheroplasts were more active in amino acid uptake, and such observations have been repeatedly encountered in incorporation studies involving cell membranes[4,5].

The antifungal agent, cycloheximide, is a specific inhibitor of 80-S ribosomal protein synthesis and is without effect on the amino acid incorporation by 70-S ribosomes from *E. coli*[29]. Moreover, cycloheximide is without effect upon bacterial growth and in this respect, *M. lysodeikticus* proved to be no exception. Cycloheximide had no growth inhibitory effect on *M. lysodeikticus* when tested at concentrations as high as 200 μg/ml nor did it inhibit the incorporation of [14C]glycine into membrane protein. Although we would agree that it seems most improbable that the chloramphenicol-resistant glycine incorporation could be due to the existence of 80-S ribosomes, this remote possibility cannot be ruled out completely until it is established whether or not the cycloheximide can pass across the permeability barrier of these bacterial cells.

DISCUSSION

Earlier studies on the nature of chloramphenicol-resistant incorporation of radioactive amino acids into bacterial cells have indicated that these could be accounted for by wall peptidoglycan synthesis[21,30], uptake into cellular lipids[21,31], and lipoamino acids, or incorporation into polypeptide antibiotics[32]. The inhibitory effect of chloramphenicol on the biosynthesis of bacterial cytoplasmic protein is well known[21]. We have also observed a rather similar level of inhibition of the synthesis of the bacterial membrane protein fraction which contains components of the electron

transport system (cytochromes and succinate dehydrogenase activity). It is perhaps striking that the part of the bacterial membrane which is affected to the greatest extent by chloramphenicol, is the 'region' of the membrane most analogous to the inner membrane of the mitochondrion and as already noted its biosynthesis is also sensitive to this drug.

From the data available at present, it appears most unlikely that the chloramphenicol-resistant incorporation into the membrane water-soluble protein fraction could in any way be related to cell-wall peptidoglycan products. Indeed, the rigorous treatments for the separation of the membrane proteins and their response to pronase, *etc.* would argue against cell-wall material as the origin of the resistant incorporation. The possibility that the membranes contain unusual polypeptides, the synthesis of which is not mediated by ribosomes, cannot be excluded at this stage.

The remote possibility that as in mitochondria, the synthesis of some of the membrane proteins could be catalysed by 80-S ribosomes, has been mentioned above. However, several other, perhaps even more plausible explanations may be invoked. It is highly possible that the biosynthesis of the chloramphenicol-insensitive membrane protein fraction is catalysed by membrane-bound ribosomes, which although similar to the cytoplasmic ribosomes, can no longer bind chloramphenicol. Inaccessibility of the chloramphenicol-inhibitable site could conceivably exist, if for example, the synthesis of certain classes of membrane proteins occurred on or in membranes or membrane vesicles *e.g.* mesosomes. The explanation of our observations must remain speculative until more is known about the sites of membrane protein synthesis in relation to the plasma or mesosome membranes of the bacterial cell and the precise enzymatic step inhibited by chloramphenicol (*e.g.* ref. 33).

It should be noted that there are several other reports of the occurrence of chloramphenicol-resistant protein synthesis in bacterial systems. KUCAN AND LIPMANN[34] found that for *E. coli*, the amino acid polymerization on endogenous messenger RNA was more resistant to chloramphenicol inhibition than polymerization occurring on a template added *in vitro*. LARK AND LARK[35] reported that the synthesis of replicator protein in *E. coli* was resistant to 30 μg chloramphenicol/ml. LEVINE AND SINSHEIMER[36] found that the synthesis of ΦX-viral-directed protein in *E. coli* was resistant to 30 μg chloramphenicol/ml but not to 100 μg/ml. This component was, moreover, associated with a rapidly sedimenting fraction which probably corresponded to 'membrane' or envelope material[36].

The results of our studies and those mentioned above[35,36] suggest that there may be distinct differences in the classes of proteins in membranes or associated with membranes and that the synthesis of these proteins may exhibit qualitative as well as quantitative differences in response to inhibitors such as chloramphenicol. The elucidation of the nature of these differences should throw further light on the biosynthesis of the membranes and the mechanism of their resistance to the inhibitory effects of chloramphenicol and related antibiotics.

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