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DIFFERENTIAL INHIBITORY EFFECTS OF CHLORAMPHENICOL ON THE SYNTHESIS OF MEMBRANE ATPase AND CYTOPLASMIC ENZYMES OF *MICROCOCCUS LYSODEIKTICUS*

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SUMMARY

When chloramphenicol (100 μg/ml) was added to cultures of *Micrococcus lysodeikticus* for about half a mean generation time, total protein was 74–78% of the control (no chloramphenicol) cultures and the levels of the cytoplasmic enzymes, polynucleotide phosphorylase (EC 2.7.7.8), adenosine deaminase (EC 3.5.4.4) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), were 74, 72–75 and 70–75%, respectively, of the amounts found in the untreated cultures. The formation of membrane-bound, Ca^{2+}-dependent ATPase (EC 3.6.1.3) of MUÑOZ *et al.*[5,6] under these conditions was unaffected by chloramphenicol. Longer periods of exposure to chloramphenicol eventually resulted in some inhibition of the formation of ATPase (85–90% of control levels) but a significant differential inhibitory effect on the synthesis of cytoplasmic enzymes persisted. The distribution of the ATPase activity between the membrane and cytoplasmic fractions was markedly affected by the protein concentration of the lysate, much of the enzyme being released into the cytoplasmic fraction at low levels of protein (0.4–2.6 mg/ml). Chloramphenicol and puromycin were without effect on the active enzyme under conditions of assay. In cultures of *Micrococcus lysodeikticus* exposed to puromycin (5 μg/ml) total protein and ATPase synthesis were affected to the same extent, being 84 and 85%, respectively, of that found for the control (no puromycin) cultures at the end of the incubation period.

INTRODUCTION

In our preceding paper[1] we observed that in the presence of chloramphenicol, growing cultures of *Micrococcus lysodeikticus* incorporated [^{14}C]glycine into a specific fraction of the membrane proteins. From these observations and those of other investigators[2,3], indicating differential sensitivities to the inhibitory effects of chloramphenicol on the synthesis of certain proteins, we thought it would be worthwhile determining the influence of chloramphenicol on the formation of some of the known enzymes of *M. lysodeikticus* of both cytoplasmic and membrane origins. The enzymatic activities selected included adenosine deaminase (EC 3.5.4.4) which had previously been shown to be a cytoplasmic enzyme in the closely related organism, *Sarcina lutea*[4], glucose-6-phosphate dehydrogenase (EC 1.1.1.49), polynucleotide phosphorylase (EC

2.7.7.8) and the membrane-bound, Ca^{2+}-dependent ATPase (EC 3.6.1.3) of *M. lysodeikticus* previously characterized in this laboratory by MUÑOZ *et al.*[5,6]. The levels of the enzymes were determined after growing *M. lysodeikticus* for about half a mean generation time (1.5 h at 30°) in the presence and absence of chloramphenicol. The results presented in this paper show that the formation of the Ca^{2+}-dependent ATPase was unaffected by chloramphenicol under conditions where the activities of all the other enzymes measured and cytoplasmic protein were significantly decreased.

MATERIALS AND METHODS

M. lysodeikticus (NCTC 2665) was grown essentially as described in the earlier paper[1]. Two cultures having identical, initial $A_{700\,m\mu}$, were grown until the $A_{700\,m\mu}$ had approximately doubled. Chloramphenicol was added to one, to give a final concentration of 100 μg/ml, and both cultures were incubated on the New Brunswick shaker for a further 1.5 h at 30°. Experiments were terminated by rapidly chilling the cultures, harvesting the cells quantitatively by centrifugation at 5° and washing the cells with cold distilled water. The cell pellets were stored overnight at 2° and the next day, cells were suspended in 25 mM Tris–HCl buffer (pH 7.5) and incubated at 37° with 500 μg lysozyme in each 8-ml sample of cell suspension, for 10–20 min or until the cells were completely lysed. Lysates were then held for an additional 15–30 min or until the viscosity visibly decreased following the addition of 250 μg deoxyribonuclease. The preparations were then centrifuged in a Model L2 Spinco centrifuge for 40 min at 67000 × *g*. The supernatant fluids (cytoplasmic fractions) were carefully decanted and held for enzyme assays, and the pellets were washed in 25 mM Tris–HCl buffer (pH 7.5) and again centrifuged as before. The supernatant, membrane-wash fractions and the cytoplasmic fractions were immediately assayed for the various enzymes. The membrane pellets were suspended in cold 50 mM Tris–HCl buffers (pH 7.5) and extracted for 20 min at 0° with 0.5 vol. of cold *n*-butanol, the aqueous and solvent phases being maintained as an emulsion by mixing with a pasteur pipette. The aqueous and solvent phases were separated by centrifugation for 20 min at 20000 × *g* and the yellow, *n*-butanol layer containing lipids and carotenoids was discarded. The clear aqueous phase (Membrane protein I) was removed for dialysis. The yellow, interfacial materials were suspended in buffer and subjected to a further extraction with *n*-butanol and the solvent phase discarded after centrifugation, as before. This second aqueous phase, together with the interfacial protein constituted "Membrane protein II". Both fractions (I and II) were dialysed overnight against 50 mM Tris–HCl buffer (pH 7.5) and then assayed for both ATPase and polynucleotide phosphorylase activities.

Enzyme assays

ATPase and polynucleotide phosphorylase were assayed as described by MUÑOZ *et al.*[5,6], except that the reactions were stopped by the addition of trichloroacetic acid, 10% (w/v) final concentration, and that the precipitated protein was removed by centrifugation. P_i was determined by the method of LOHMANN AND JENDRASSIK[7]. Adenosine deaminase was assayed as described by ROBRISH AND MARR[8]. Glucose-6-phosphate dehydrogenase was determined spectrophotometrically[9].

Proteins were determined by the method of LOWRY *et al.*[10] using bovine serum

albumin as a standard. Proteins in the cytoplasmic and membrane-wash fractions were usually first precipitated with 10 % trichloroacetic acid (final concentration) and dissolved in 0.25 M NaOH and 0.2 % sodium dodecylsulfate when needed.

Chloramphenicol, Sigma Chemical Co., St. Louis, Mo., was used throughout these studies.

RESULTS

In order to determine the effect of chloramphenicol on the synthesis of *M. lysodeikticus* membrane and cytoplasmic enzymes, two cultures were prepared and grown under conditions which were, as far as possible, identical. Chloramphenicol was added to one of the two as described in MATERIALS AND METHODS. Although care was taken to keep the growth rate of both cultures as similar as possible, it should be noted that upon addition of the chloramphenicol, the $A_{700\,m\mu}$ of the culture containing the drug invariably increased more rapidly than that of the control (no chloramphenicol) as shown in Fig. 1. The precise reasons for this fairly rapid increase in $A_{700\,m\mu}$ are unknown but may conceivably be related to cell swelling, increased wall thickness or even increased membrane synthesis.

After the cells were harvested, lysed and the lysates fractionated, the fractions were assayed for Ca^{2+}-dependent ATPase, polynucleotide phosphorylase, adenosine deaminase and glucose-6-phosphate dehydrogenase activities and for their protein contents. Preliminary experiments had established that virtually all of the adenosine deaminase and glucose-6-phosphate dehydrogenase activities were in the cytoplasmic fractions. The results presented in Table I show that chloramphenicol had no inhibitory effect on the formation of ATPase as determined by the total activity in all fractions, whereas the synthesis of other enzymes was inhibited and the levels of the enzymes were 70–74 % of those observed for control (no chloramphenicol) cultures. As shown in Table I total protein under these conditions of growth for 1.5 h at 30° in the presence of chloramphenicol (100 μg/ml) was 78 % (Expt. I) and 74 % (Expt. II) of the control levels. However, it should be noted that in earlier preliminary experiments, exposure to chloramphenicol (100 μg/ml) for 3 h (about a mean generation time based on $A_{700\,m\mu}$ increase) resulted in approx. 10–15 % lowering of the ATPase level (relative to control enzyme assays) compared to a 35–40 % decrease in the amounts of cytoplasmic enzymes present in the treated cultures. As the ATPase assays in the preliminary experiments were performed before we had discovered the influence of lysate protein concentration on release of active enzyme, it is likely that the real inhibitory effects of chloramphenicol would be less.

That the differential inhibitory effects of chloramphenicol observed on the formation of ATPase and cytoplasmic enzymes were not due to the cessation of synthesis of certain enzymes during this phase of growth of *M. lysodeikticus* was confirmed by assaying ATPase and polynucleotide phosphorylase activities over the 1.5-h growth period. Units of ATPase activity rose from an initial value of 34.7 to a final level of 52.8 units in the control cultures with a final level of 47.9 in the chloramphenicol-treated culture. On the other hand polynucleotide phosphorylase had an initial activity of 115 units and a final level of 150 units and the culture containing chloramphenicol (100 μg/ml) showed complete inhibition of enzyme formation (115 units).

Although MUÑOZ *et al.*[5,6] found that the "latent" ATPase was bound to the membrane of *M. lysodeikticus* grown and isolated under "standard" conditions de-

veloped in this laboratory[11], ATPase activity could be released to a great extent by reducing the ionic strength of the wash buffer[5]. The influence of such factors as different media and growth conditions on the distribution and release of membrane enzymes of *M. lysodeikticus* has not been investigated. It is, however, apparent from a comparison of the results obtained with cells grown under "standard" conditions[5,6] with those from the diluted peptone–water–yeast extract medium used in the present studies and the preceding paper[1], that differences in the pattern of release of ATPase result. This difference in growth conditions, together with the absence of Mg^{2+} in the initial lysis medium, probably affects the stability of the membranes and accounts for the appearance of so much of the ATPase activity in the cytoplasmic and membrane-wash fractions as shown in Table I. It has also been observed with the isolated *M. lysodeikticus* ATPase that upon storage or upon passage through Sephadex it increased in activity. This behavior was somewhat similar to the latent properties exhibited by spinach chloroplast ATPase reported by VAMBUTAS AND RACKER[12]. It was conceivable, therefore, that the apparent lack of inhibition of synthesis by chloramphenicol could be due to the chloramphenicol-treated culture of *M. lysodeikticus* containing more of the activated form of ATPase and less latent enzyme than the control culture. For this reason the ATPase in all fractions was assayed after storage at −20° for 7 days in Expt. II and 15 days in Expt. I. The results given in Table I do not indicate that there is less total ATPase, either latent or active form, in the fractions from the cells grown in the presence of chloramphenicol. However, the data do suggest that on prolonged storage (15 days) ATPase activities of the fractions from the chloramphenicol-treated cells decreased more slowly than those of the untreated controls.

A comparison of Expts. I and II in Table I indicates that although the total protein was higher in the latter experiment, the total number of initial units of ATPase activity was rather less. This suggested the possibility that lysate protein concentration may influence the release of ATPase activity and accordingly *M. lysodeikticus* cells were lysed under "dilute" and "concentrated" conditions. The results summarized in Table II show that more active ATPase, with a higher specific activity was obtained when the same cells were lysed under dilute conditions. In view of this marked effect of lysate protein concentration upon the levels of ATPase released from the membranes, the effect of chloramphenicol treatment was determined for cells lysed under dilute conditions. The results summarized in Table III show that nearly all of the ATPase was released under such conditions of lysis and that a substantially larger amount was in the active form although some latent enzyme was still present. After storage for 1 day, ATPase activity increased substantially (*i.e.* 2nd day assay, Table III) but upon further storage, enzyme inactivation occurred.

Both on the 1st and 2nd day assays under dilute lysis conditions, the fractions from the chloramphenicol-treated cells showed ATPase activities 11–15 % higher than those of the cells grown in the absence of the antibiotic. That this stimulation of activity was not due to a direct effect on the enzyme was shown by assaying the fractions in the presence of chloramphenicol as presented in Table IV. Chloramphenicol neither stimulated nor inhibited the activity under conditions of the assay. As the level of activity in the absence of added Ca^{2+} was about the same for both control and chloramphenicol-treated fractions, it appeared unlikely that the higher activity reported in Table III could be due to formation of a nonspecific phosphatase.

TABLE I

EFFECT OF CHLORAMPHENICOL ON ENZYME AND PROTEIN LEVELS OF *M. lysodeikticus*

Expt. I: Two cultures, 560 ml each, with initial $A_{700\,m\mu} = 0.160$, were grown for about 3 h to $A_{700\,m\mu} = 0.400$. Chloramphenicol (100 μg/ml, final concentration) was added to one and both cultures were grown for another 1.5 h (final $A_{700\,m\mu}$ of control, 0.549; + chloramphenicol, 0.585). Cultures were chilled, lysed, lysates fractionated and fractions assayed for enzymes as described in MATERIALS AND METHODS. Conditions in Expt. II as in Expt. I, except that culture volume was 600 ml, initial $A_{700\,m\mu} = 0.175$, both grown to $A_{700\,m\mu} = 0.410$ and the final $A_{700\,m\mu}$'s after 1.5 h growth were 0.540 and 0.591 for control (without chloramphenicol) and chloramphenicol cultures, respectively.

Cell fraction	*Protein (mg/fraction)*	*ATPase (units/fraction)*		*Specific activity*	*Polynucleotide phosphorylase (units/fraction)*	*Adenosine deaminase (units/fraction)*	*Glucose-6-phosphatase dehydrogenase (units/fraction)*
Expt. I ***							
Control							
Cytoplasmic	57.1	47.2	(14.6) *	0.8	274	28.3	2.65
Membrane wash	2.6	3.9		1.5	8.8	—	
n-Butanol extracted							
membrane protein I	1.12	11.3	(2.20) *	10.0	27.5	—	
membrane protein II	0.57	0			0.95		
Totals	61.39	62.4			311.25	28.3	2.65
Chloramphenicol							
Cytoplasmic	43.2	47.4	(16.6) *	1.1	201	20.3	1.86
Membrane wash	2.9	7.1		2.5	5.8	—	—
n-Butanol extracted							
membrane protein I	1.4	9.0	(2.22) *	6.4	21.4	—	—
membrane protein II	0.5	0			0.24		
Totals	48.03	63.5			228.44	20.3	1.86
% of control	78	102			74	72	70
Expt. II ***							
Control							
Cytoplasmic	65.0	28.6	(41.6) **	0.44	366	34.25	3.52
Membrane wash	7.17	6.0	(2.9) **	0.84	32.5	2.56	—

n-Butanol extracted						
membrane protein I	2.26	11.6 (8.2)**	5.1	7.7	0	—
membrane protein II	0.57	0.95 (0.6)**	1.7	0.63		
Totals	75.00	47.15 (53.3)**		406.83	36.81	3.52
Chloramphenicol						
Cytoplasmic	45.6	28.3 (46.2)**	0.62	266	25.7	2.63
Membrane wash	6.53	7.78 (3.9)**	1.2	29.3	1.8	—
n-Butanol extracted						
membrane protein I	2.86	9.2 (12.3)**	3.2	5.7	0	—
membrane protein II	0.64	0.43 (0.47)**	0.67	0.27		
Totals	55.60	45.71 (62.8)**		301.27	27.5	2.63
% of control	74	97 (>100)		74	75	75

* Enzyme assayed after 15-day storage at −20°.
** Enzyme assayed after 7-day storage at −20°.
*** Expt. I, lysate proteins: 2.2 mg/ml (control); 1.7 mg/ml (+ chloramphenicol). Expt. II, lysate proteins: 2.6 mg/ml (control); 1.9 mg/ml (+ chloramphenicol).

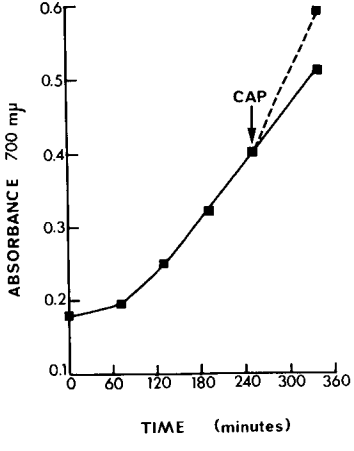

Fig. 1. Growth of *M. lysodeikticus* on diluted (1:8) peptone–water–yeast extract medium at 30°, indicating the stage at which chloramphenicol (CAP), 100 μg/ml final concentration, was added to one of two identical cultures. The upper curve (– – – –) for the culture containing chloramphenicol shows the increase in absorbance above the level of the control (no chloramphenicol) culture.

Puromycin had been found to be an inhibitor of [^{14}C]glycine incorporation into both cytoplasmic and membrane protein fractions in our earlier study[1]. It was, therefore, of interest to determine its effect on the synthesis of ATPase by *M. lysodeikticus*. The data summarized in Table V show that puromycin inhibited total protein synthesis, membrane protein synthesis and ATPase activity to about the same extent. Upon storage of the fractions from the puromycin-inhibited cells, the

TABLE II

ATPase ACTIVITY OF DILUTE AND CONCENTRATED *M. lysodeikticus* LYSATES

An exponentially growing culture was divided into two equal portions. One-half of washed bacteria was suspended in 25 mM Tris buffer (pH 7.5) to give a final $A_{700\,m\mu}$ of approx. 10.0. The other half of the culture was suspended in 4 times as much buffer, to give an approximate final $A_{700\,m\mu}$ of 2.50. Both were lysed, centrifuged and assayed for ATPase activity as described in MATERIALS AND METHODS.

Cell fraction	*Protein (mg/ml)*	*ATPase*		
		Units/ml	*Units/fraction*	*Specific activity*
Cytoplasmic	0.41	0.95	30.4	2.3
Cytoplasmic	1.72	2.13	17.0	1.2

TABLE III

ATPase ACTIVITY OF CONTROL AND CHLORAMPHENICOL-TREATED CULTURES OF *M. lysodeikticus* LYSED UNDER DILUTE CONDITIONS

Initial $A_{700\,m\mu}$ of each 240-ml culture was 0.180. Chloramphenicol was added to one when both had reached $A_{700\,m\mu}$ = 0.400; both were grown for 1.5 h longer (final $A_{700\,m\mu}$: control, 0.510; + chloramphenicol, 0.592). Cells harvested from each culture were suspended in 40 ml of 25 mM Tris buffer (pH 7.5) lysed and fractionated as described in MATERIALS AND METHODS.

Cell fraction	*Protein (mg/fraction)*	*ATPase activity*		
		1st day (units/fraction)	*2nd day (units/fraction)*	*Specific activity*
Control				
Cytoplasmic*	19.94	29.7	40.6	2.0
Membrane wash	0.34	1.8	1.8	
n-Butanol extracted membrane protein	0.81	0.8	0.8	2.0
Totals	21.09	32.3	43.2	
Chloramphenicol				
Cytoplasmic**	13.78	33.4	48.6	3.5
Membrane wash	0.39	1.9	1.9	
n-Butanol extracted membrane protein	1.33	0.7	0.7	0.5
Totals	15.50	36.0	51.2	
% of control	74	111***	115***	

* Fraction contained 0.50 mg/ml.
** Fraction contained 0.35 mg/ml.
*** *i.e.* "stimulation" of activity.

TABLE IV

CHLORAMPHENICOL EFFECT ON THE ATPase ASSAY SYSTEM

Cell fraction	*ATPase*	
	Assay system	*Units/fraction*
Control, cytoplasmic	Complete	31.0
	+ 70 μg chloramphenicol	31.0
	− Ca^{2+}	8.2
Chloramphenicol, cytoplasmic	Complete	31.4
	+ 70 μg chloramphenicol	31.0
	− Ca^{2+}	7.3

TABLE V

ATPase ACTIVITY OF CONTROL AND PUROMYCIN-TREATED CULTURES OF *M. lysodeikticus* LYSED UNDER DILUTE CONDITIONS

Two cultures, 150 ml each, with initial $A_{700\,m\mu}$ = 0.161 were grown to $A_{700\,m\mu}$ = 0.352. Puromycin (5 μg/ml, final concentration) was added to one and both cultures incubated for an additional 2 h. Final $A_{700\,m\mu}$'s were 0.580 for control and 0.512 for puromycin-treated cultures. Cells were harvested, lysed and fractionated as described for experiment in Table III.

Cell fraction	*Protein (mg/fraction)*	*ATPase activity*		
		1st day (units/fraction)	*Specific activity*	*4th day (units/fraction)*
Control				
cytoplasmic*	15.15	28.8	1.9	15.5
membrane wash	2.57	1.3	0.52	1.6
membrane	2.98	—	0.23	0.7
Totals	20.70	30.1		17.8
Puromycin**				
cytoplasmic	12.40	23.8	1.9	10.1
membrane wash	2.36	1.1	0.47	1.1
membrane	2.74	—	0.20	0.56
Totals	17.50	25.5		11.76
% of control	84	85		76

* Fraction contained 0.51 mg protein per ml.
** Fraction contained 0.42 mg protein per ml.

ATPase activity decreased even more rapidly, but the specific activities of fractions did not differ significantly from those of the cultures grown in the absence of puromycin. As in the case of chloramphenicol (Table IV), puromycin at a level of 6 μg/0.5 ml had no effect on the activity of the ATPase (μmoles P_i released/10 min were 0.35 for control and 0.34 for puromycin-treated fractions), thus suggesting an inhibitory action on synthesis rather than an effect on assembly of preexisting enzyme subunits.

DISCUSSION

Differences in the sensitivity of the synthesis of certain proteins to the inhibitory effects of chloramphenicol has been reported for the replicator protein by LARK AND

LARK[2] and the synthesis of a viral-directed protein by LEVINE AND SINSHEIMER[3], both in *Escherichia coli*. The investigations presented in this paper extend this chloramphenicol-resistant synthesis of protein to the Ca^{2+}-dependent adenosine triphosphatase of *M. lysodeikticus*. It is perhaps significant that in all three instances exhibiting this phenomenon, proteins having some degree of association with the membranes have been involved. The mechanism of this resistance to chloramphenicol is obscure but it is of interest to note that KUCAN AND LIPMANN[13] found that amino acid polymerization involving endogenous messenger RNA in *E. coli* was more resistant than that occurring on a template added *in vitro*. Whether these observations can be explained by qualitative or quantitative differences in the chloramphenicol-binding site on ribosomes or accessibility of different classes of ribosomes to the antibiotic can only be answered by further investigations.

As the *M. lysodeikticus* ATPase is composed of six peripheral subunits surrounding a central unit[14], it seemed conceivable that the lack of inhibitory effect of chloramphenicol on total activity in the various fractions could have been due to the continued assembly of the enzyme from a hypothetical pool of inactive subunits to yield active enzyme molecules. Thus, the assembly of the enzymatically active ATPase particle[14] could be insensitive to chloramphenicol, but the synthesis of new subunits would be sensitive to the drug. However, the fact that puromycin inhibits the formation of ATPase to the same extent as total protein synthesis, argues strongly against the above suggestion of the existence of a pool of inactive subunits and continued assembly in the presence of chloramphenicol.

The evidence thus far available suggests ribosomal involvement in this phenomenon and the elucidation of the resistance to chloramphenicol may yield further information about the control and mechanism of synthesis of certain membrane proteins. It is likely from our previous study on the incorporation of [^{14}C]glycine into the membrane proteins[1], that proteins other than the ATPase also exhibit this phenomenon. In their recent studies of *Mycoplasma laidlawii* membranes KAHANE AND RAZIN[15] did not observe any chloramphenicol-resistant incorporation of [^{14}C]phenylalanine into membrane protein but they did note, without further comment or evidence, that ATPase activity was unaffected. There seems little doubt that there will be marked differences between bacterial species in their response to the inhibitory effects of chloramphenicol on the synthesis of specific membrane proteins.

In view of our previous discovery that the ATPase was largely localized on the membrane prepared under our standard conditions[5,6,11], we were surprised to find that much of the activity was released into the cytoplasmic fraction from the cells grown on the diluted peptone–water–yeast extract medium used in the present studies. It is quite possible that the stability of the membranes is involved, a suspicion that is reinforced by carrying out the lysis under more dilute conditions as shown in Tables II and III. Upon lysis of dilute suspensions very little ATPase and very little proteins is left in the membrane fraction after *n*-butanol extraction (Tables III and IV). It therefore appears likely that the stability of the membrane is increased with increasing total protein concentration of the lysate. Under our standard conditions of isolating the membranes in quantity[5,6,11], the protein levels of the lysates were about 5–10 times those of the present work (*i.e.* approx. 10 mg/ml). The influence of the divalent cation status of the diluted peptone–water–yeast extract medium and the effects of cell suspension density and protein concentration during lysis upon the

stability of the membranes obviously require further investigation. The effects of such factors on the stability of the membrane will have to be controlled before we can determine what fraction of the chloramphenicol-resistant [^{14}C]glycine incorporation is due to the continued synthesis of the ATPase. The inhibition of cytoplasmic protein synthesis by chloramphenicol, based upon [^{14}C]glycine incorporation was 90–97 % (ref. 1) and some of this 3–10 % residual synthesis could be due to released ATPase. It is worth noting that even if all the membrane ATPase had been released into the cytoplasmic fraction it would only account for about 2 % of the total protein, based on our previous calculations that it accounts for approx. 10 % of the total membrane protein[6].

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