Biochemical Pharmacology, 1962, Vol. 11, pp. 829-833. Pergamon Press Ltd., Printed in Great Britain.

PENICILLIN-INDUCED SPHEROPLAST FORMATION IN SYNTHETIC MEDIA

W. B. HUGO* and A. D. RUSSELL†

*Department of Pharmacy, University of Nottingham, England, and
†Welsh School of Pharmacy, Welsh College of Advanced Technology, Cardiff, Wales

(*Received* 1 *March* 1962; *accepted* 24 *May* 1962)

Abstract—The induction of spheroplasts of *Escherichia coli* by treatment of the organism with penicillin in a simple, chemically defined medium is described, and is shown to depend on the available concentration of Mg^{2+} ions, on the temperature of incubation, and on the stage of growth of the bacterial cells when penicillin is added.

INTRODUCTION

A SURVEY of the literature indicates that information on the induction of spheroplasts in synthetic media is incomplete. Lark[1] induced spheroplasts in *Alcaligenes faecalis* by treating the organism with penicillin in a medium containing salts and glutamic acid or casamino acids (vitamin-free; Difco), while Lederberg and St. Clair[2] reported a slow and incomplete formation of spheroplasts when *Escherichia coli* was treated with penicillin in a medium[3] consisting of salts and glucose. An attempt at inducing spheroplasts of *Aerobacter aerogenes* by penicillin treatment in a synthetic medium containing high concentrations of sucrose proved to be unsuccessful.[4, 5]

In this paper aspects of the action of penicillin on *Escherichia coli* in synthetic medium is described.

MEDIA AND METHODS

The organism was *E. coli*, Type I, formerly NCTC 5934. Viable counts were made by serial dilution and plating. Penicillinase was included in the first dilution tube to destroy any residual penicillin activity. Such a procedure resulted in the explosion of spheroplasts so that no colonies could have been derived from the spheroplast regrowth. Total spheroplast counts were made with a Helber or Thoma counting chamber using interference microscopy. Culture density was measured by a nephelometer (Evans Electroselenium Ltd., Harlow).

All chemicals were of Analytical Reagent quality, except sodium lactate, which was of Laboratory Reagent grade.

Media used throughout this work were of the following composition:

Nutrient broth. Peptone (Oxoid) 10 g, Lab Lemco (Oxoid) 10 g, sodium chloride 5 g, water to 1 l. Final pH after sterilization was 7·2.

Synthetic medium A[6]. $(NH_4)_2HPO_4$ 0·3144 g, NaCl 0·1 g, $FeSO_4 \cdot 7H_2O$ 0·03 g, KH_2PO_4 0·04 g, $MgSO_4 \cdot 7H_2O$ 0·07 g, water to 100 ml. pH after sterilization was adjusted to 7·2.

Synthetic medium B. The following medium was evolved to avoid the precipitation which occurred in A: $(NH_4)_2 HPO_4$ 0·06 g, NaCl 0·1 g, $FeSO_4 \cdot 7H_2O$ 0·001 g, KH_2PO_4 0·04 g, $MgSO_4 \cdot 7H_2O$ as required, water to 100 ml, pH 7·2.

The water for each medium was distilled twice in an all-glass still. Utilizable carbon source was 2% w/v glucose, glycerol or sodium lactate. Sucrose was present (0·33 M) or absent.

Washed suspensions of the organism were prepared by growing 10 ml quantities of the bacteria in nutrient broth for 17 hr on a rotator at 37 °C, centrifuging, and washing the residue twice with 5 ml of sterile water.

RESULTS

Growth in medium A

A volume of 0·5 ml of the washed suspension was added to 10 ml quantities of media, and incubating at 37 °C. The results (Table 1) indicate that the growth obtained when glucose or glycerol formed the carbon source could be further stimulated by the addition of 0·1% w/v $MgSO_4 \cdot 7H_2O$ over and above that concentration of $MgSO_4 \cdot 7H_2O$ which might remain in the filtrate obtained in the preparation of the medium.

TABLE 1. GROWTH OF *E. Coli* IN SYNTHETIC MEDIUM A CONTAINING 0·33 M SUCROSE

Carbon source (2% w/v)	Presence (+) or absence (−) of extra 0·1% w/v $MgSO_4 \cdot 7H_2O$	Viable count/ml 0 hr	Viable count/ml 5 hr at 37 °C
Glucose	−	3×10^7	$8{\cdot}1 \times 10^7$
	+	3×10^7	1×10^8
Glycerol	−	3×10^7	$1{\cdot}4 \times 10^8$
	+	3×10^7	$1{\cdot}8 \times 10^8$
Lactate	−	3×10^7	$4{\cdot}5 \times 10^7$
	+	3×10^7	$4{\cdot}5 \times 10^7$

Spheroplast formation in medium A

The induction of spheroplasts by treatment of the organism with 5000 units/ml showed that it was necessary to transfer the medium to room temperature (18 °C) after a preliminary incubation at 37 °C, to ensure optimum spheroplast stability (Table 2).

TABLE 2. INDUCTION OF SPHEROPLASTS IN MEDIUM A

Carbon source (2% w/v)	Presence (+) or absence (−) of added 0·1% w/v $MgSO_4 \cdot 7H_2O$	Viable count/ml at 37 °C		Spheroplasts/ml after 24 hr	
		5 hr	24 hr	37 °C*	18 °C†
Glucose	+	$7{\cdot}9 \times 10^5$	$2{\cdot}3 \times 10^3$	$1{\cdot}5 \times 10^6$	$3{\cdot}6 \times 10^6$
		n.t.	n.t.	$<10^6$	$2{\cdot}5 \times 10^6$
Glycerol	+	$1{\cdot}2 \times 10^5$	7×10^2	$1{\cdot}5 \times 10^6$	$7{\cdot}8 \times 10^6$
	−	n.t.	n.t.	$<10^6$	3×10^6
Lactate	+	$1{\cdot}6 \times 10^7$	$7{\cdot}5 \times 10^3$	$<10^6$	$<10^6$
	−	n.t.	n.t.	$<10^6$	$<10^6$

Viable count/ml at 0 hr = $3{\cdot}2 \times 10^7$.
* 24 hr at 37 °C.
† 5 hr at 37 °C, then 19 hr at 18 °C.
n.t. not tested.
Penicillin concn. 5000 units/ml.

A study of the effect of temperature on the stability of *E. coli* spheroplasts induced by treatment of the organism with 5000 units/ml penicillin in a nutrient broth conversion medium has indicated that spheroplasts were better preserved at temperatures below the optimum for growth.[7]

Table 2 also shows that an additional source of Mg^{2+} ions is necessary for the stability of spheroplasts.

Growth in medium B

The growth of *E. coli* in medium B gave the results shown in Fig. 1, where the need for the presence of Mg^{2+} ions is again apparent. Optimum growth was obtained with glucose as utilizable carbon source. The high osmotic pressure of the medium when sucrose was included retarded the rate of, but did not prevent, growth.

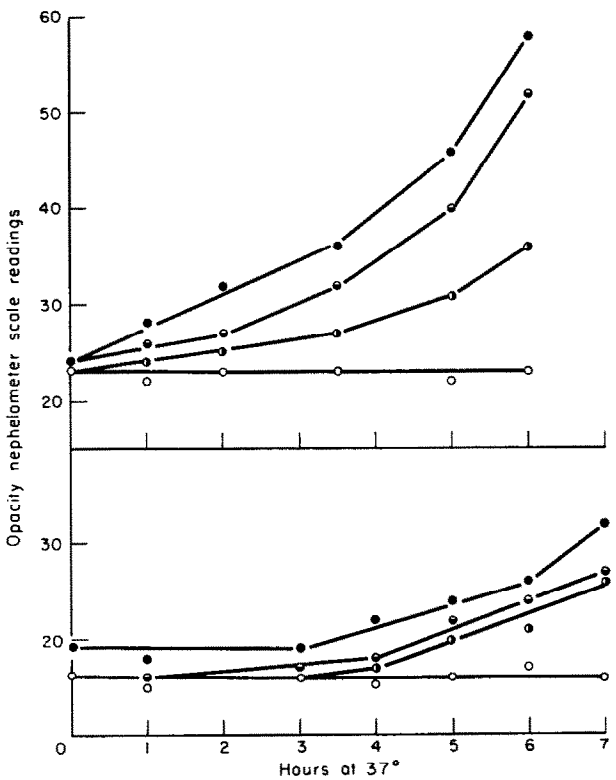

FIG. 1. Effect of $MgSO_4 \cdot 7H_2O$ concentration on the growth of *E. coli* in synthetic medium B.

Upper portion: sucrose absent. Lower portion: 0·33 M sucrose present.
Absent ○——○ 0·005% w/v ◑——◑.
0·05% w/v ◒——◒ 0·1% w/v ●——●.

Spheroplast formation in medium B

The induction of spheroplasts in this medium containing 5000 units/ml penicillin is shown in Table 3. A preliminary experiment had shown that a short exposure (1–5 hr) at 37° prior to incubation at 18 °C gave optimum spheroplast formation.

In all previous spheroplast-inducing experiments, the antibiotic was added to the bacterial cells at 0 min. The effect of adding the penicillin (to give 5000 units/ml) at a point when the bacteria have started to divide is shown in Table 4, the results of which should be compared with those of Table 3.

TABLE 3. EFFECT OF $MgSO_4.7H_2O$ CONCENTRATION ON SPHEROPLAST FORMATION IN MEDIUM B

$MgSO_4.7H_2O$ concn. (% w/v)	Viable count/ml 5 hr at 37 °C	Counts/ml after further 19 hr at 18 °C	
		Viable	Spheroplast
0	3×10^6	6×10^5	0.1×10^6
0·005	$2{\cdot}6 \times 10^6$	6×10^4	$2{\cdot}3 \times 10^6$
0·01	7×10^5	$4{\cdot}7 \times 10^4$	$3{\cdot}8 \times 10^6$
0·02	$8{\cdot}3 \times 10^5$	$4{\cdot}2 \times 10^4$	$4{\cdot}6 \times 10^6$
0·05	$6{\cdot}6 \times 10^5$	$2{\cdot}9 \times 10^4$	$5{\cdot}5 \times 10^6$
0·10	$6{\cdot}3 \times 10^5$	$2{\cdot}6 \times 10^4$	4×10^6

Viable count/ml at 0 hr = $1{\cdot}4 \times 10^7$.
Penicillin concn. 5000 units/ml.

TABLE 4. EFFECT OF ADDING 5000 UNITS/ML PENICILLIN 4 HR AFTER COMMENCEMENT OF INCUBATION AT 37 °C USING AN ORIGINAL INOCULUM OF $3{\cdot}2 \times 10^7$ VIABLE CELLS/ML

$MgSO_4.7H_2O$ concn. (% w/v)	Viable count/ml when penicillin added (i.e. after 4 hr 37 °C)	Continued incubation for 20 hr at			
		18 °C		37 °C	
		Viable cells/ml	Spheres/ml	Viable cells/ml	Spheres/ml
0·005	$2{\cdot}8 \times 10^7$	$1{\cdot}3 \times 10^6$	$3{\cdot}2 \times 10^6$	—	c.10^6
0·01	—	$1{\cdot}8 \times 10^5$	$9{\cdot}4 \times 10^6$	—	9×10^6
0·05	9×10^7	$4{\cdot}3 \times 10^5$	$1{\cdot}5 \times 10^7$	—	$2{\cdot}3 \times 10^7$
0·10	8×10^7	$1{\cdot}1 \times 10^6$	$3{\cdot}8 \times 10^7$*	4×10^3	$2{\cdot}9 \times 10^7$

* Difficult in several cases to distinguish spheroplasts and rods, thus count may be somewhat higher than it should be.

DISCUSSION

The induction of spheroplasts of *E. coli* by treatment of the organism with penicillin in synthetic media appears to depend on three factors: firstly, the presence of Mg^{2+} ions is essential not only for the growth of the organism in penicillin-free medium but also for the stability of the spheroplasts induced by penicillin action. The stability of spheroplasts in a conventional nutrient medium is known to depend partly on a readily available source of Mg^{2+} ions[2, 8, 9] which presumably stabilize the cytoplasmic membrane.[10] The replacement of Mg^{2+} by Ca^{2+} gave similar results in nutrient broth (Lederberg[8]), and in this medium we have found it possible to replace Mg^{2+} and Ca^{2+} by Sr^{2+} and Ba^{2+}. In our synthetic medium B, Mn^{2+}, Be^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} gave precipitation and thus could not be used; secondly, the temperature of incubation plays an important part in the number of spheroplasts observed; thirdly, the stage of growth of the bacterial cells must be considered. Higher spheroplast yields could be obtained when the penicillin was added to cells which were in an active state

of cell division (Table 4) than when added to bacteria which had not previously been allowed to divide (Table 3). New cell wall material is presumably being built up at a rapid rate in the former case in comparison to the latter system, and as the main antibacterial mechanism of penicillin action is to inhibit cell wall synthesis, it would be expected that the antibiotic exerts this effect on actively dividing cells more than on bacteria which had not been given the opportunity to divide before treatment with this substance.

Spheroplasts of *E. coli* can be induced within the first 2 hr incubation at 37 °C in a conventional nutrient conversion medium,[9] which is far more rapid than in the synthetic media used in the present report, although the final conversion of rods into spheroplasts is of a similar order. It is of interest to record here the finding by Schwartzman[11] that the susceptibility of some Gram-negative bacteria towards penicillin was significantly greater in synthetic medium than in meat infusion broth.

REFERENCES

1. K. G. Lark, *Canad. J. Microbiol.* **4**, 165 (1958).
2. J. Lederberg and J. St. Clair, *J. Bact.* **75**, 143 (1958).
3. C. H. Gray and E. L. Tatum, *Proc. Nat. Acad. Sci., Wash.* **30**, 404 (1944).
4. J. M. Gebicki and A. M. James, *Nature, Lond.* **182**, 725 (1958).
5. J. M. Gebicki and A. M. James, *J. Gen. Microbiol.* **23**, 9 (1960).
6. E. F. Gale, *The Chemical Activities of Bacteria* p. 176. University Tutorial Press, London (1947).
7. W. B. Hugo and A. D. Russell, *J. Pharm., Lond.* **13**, 688 (1961).
8. J. Lederberg, *Proc. Nat. Acad. Sci., Wash.* **42**, 574 (1956).
9. W. B. Hugo and A. D. Russell, *J. Bact.* **80**, 436 (1960).
10. K. McQuillen, In *The Bacteria* (Edited by I. C. Gunsalus and R. Y. Stanier) Vol. 1. Academic Press, New York and London (1959).
11. G. Schwartzman, *Science* **101**, 276 (1945).