Phytochemistry, 1967, Vol. 6, pp. 799 to 806. Pergamon Press Ltd. Printed in England

EFFECTS OF ANTIBIOTICS ON PROTEIN AND RIBONUCLEIC ACID SYNTHESIS IN RELATION TO THE CONTROL OF BACTERIAL GROWTH IN EXCISED TISSUES

M. A. VENIS

Research Institute, Canada Agriculture, University Sub P.O., London, Ontario, Canada

(*Received* 30 *November* 1966)

Abstract—Penicillin in the range 15–1000 mg/l. is generally without effect on incorporation of ^{14}C-leucine into protein or of ^{14}C-adenine into ribonucleic acid by excised pea and cucumber segments. After 16 hr pretreatment in 1000 mg/l. penicillin, leucine incorporation is depressed somewhat. Incubation in sterile buffer supplemented with penicillin gives considerable control of bacterial growth within the tissues. Streptomycin strongly inhibits protein synthesis, producing 50–75 per cent inhibition at 50 mg/l. Adenine incorporation is affected to a lesser extent. Evidence based on the differential effects of penicillin and of cycloheximide on leucine incorporation and on bacterial growth indicates that even high levels of bacterial contamination, obtained in tissues incubated without penicillin, do not contribute significantly to the measured rates of incorporation.

INTRODUCTION

WILSON[1] has recently emphasized the importance of minimizing bacterial contamination during studies with ribosomal incorporation systems. For work with such *in vitro* systems the problem is readily surmounted by using sterile buffers for homogenization and for suspension of fractions. A more difficult problem arises when investigating *in vivo* incorporation by excised plant tissues. In the absence of facilities for growing plants in quantity under completely aseptic conditions, many workers resort to the inclusion of antibiotics in incubation media. Streptomycin and penicillin, singly or in combination, are most commonly used, the assumption being that at the concentrations employed the antibiotics are without effect on the incorporation process under study. However, this assumption is seldom checked and there are few data in the literature to serve as a guide. The inhibition of chlorophyll development by high concentrations of streptomycin is well known.[2] At similar concentrations (600–4000 mg/l.) streptomycin has also been reported to inhibit both protein and RNA synthesis in tobacco leaves[3] and in *Euglena gracilis*.[4] On the other hand, a much lower concentration of dihydrostreptomycin (50 mg/l.) inhibited neither uracil nor leucine incorporation in potato slices, even over a 24-hr period.[5] Amino acid incorporation by isolated plant ribosomes has been found to be either sensitive[6] or insensitive[7] to low concentrations of streptomycin in different systems. The only similar information for penicillin appears to be Parthier's report[3] that 8×10^{-4} M penicillin G was without effect on RNA or protein synthesis *in vivo*.

[1] C. W. WILSON, *Plant Physiol.* **41**, 325 (1966).
[2] H. VON EULER, M. BRACCO and L. HELLER, *Compt. Rend.* **227**, 16 (1948).
[3] B. PARTHIER, *Nature* **206**, 783 (1965).
[4] J. T. O. KIRK, *Biochem. Biophys. Acta* **56**, 139 (1962).
[5] G. G. LATIES, *Plant Physiol.* **40**, 1237 (1965).
[6] A. A. APP and L. U. BARTON, *Contrib. Boyce Thompson Inst.* **23**, 127 (1965).
[7] B. PARISI and O. CIFERRI, *Biochemistry* **5**, 1638 (1966).

In view of the few and somewhat conflicting data available, an investigation has been undertaken to study the effects of streptomycin and penicillin on protein and RNA synthesis in two excised tissues used in this laboratory, the pea epicotyl and the cucumber hypocotyl. In addition, the effectiveness of penicillin in controlling the endogenous bacterial populations of these tissues has been studied.

RESULTS

Initially, the extent of bacterial contamination of pea segments under the worst possible conditions was examined, that is following incubation in non-sterile buffers without antibiotics. The bacterial population of fresh tissue is fairly low, rises six-fold after 3·5 hr and reaches severe proportions after 17-hr incubation (Table 1). Considerable control is effected by the inclusion of penicillin in the non-sterile buffer (Table 2). From Table 3 it can be seen

TABLE 1. THE EFFECT OF INCUBATION IN NON-STERILE BUFFER ON THE BACTERIAL POPULATION OF PEA SEGMENTS

Incubation time (hr)	Colonies/g tissue
0	8×10^3
3·5	5×10^4
17	$1{\cdot}6 \times 10^8$

TABLE 2. THE EFFECT OF PENICILLIN ON BACTERIAL GROWTH IN PEA SEGMENTS INCUBATED IN NON-STERILE BUFFER

Concn. of penicillin (mg/l.)	Incubation time (hr)	Colonies/g tissue ($\times 10^{-3}$)
—	0	3·6
15	4·5	9
1000	4·5	7
15	17	540
1000	17	30

TABLE 3. THE EFFECT OF INCUBATION IN STERILE BUFFER, WITH OR WITHOUT 100 MG/L. PENICILLIN, ON BACTERIAL GROWTH IN PEA AND CUCUMBER SEGMENTS

Medium	Incubation time (hr)	Colonies/g tissue, $\times 10^{-3}$	
		Pea	Cucumber
—	0	30	200
Buffer	4	60	500
Penicillin	4	1·2	5
Buffer	18	8000	20,000
Penicillin	18	60	2000

that when sterilized buffer is used for incubation, together with 100 mg/l. penicillin, the bacterial counts of both pea and cucumber segments are very low after 4 hr (despite the higher initial contamination of the tissue in this experiment—cf. Tables 1 and 2). Even at the end of 18 hr under these conditions, contamination of pea segments is not severe, although a high count is obtained from cucumber tissue after this time. It will be noted from Table 3 that for both tissues contamination after 4 hr in 100 mg/l. penicillin is considerably less than that of freshly-excised segments, whereas when non-sterile buffer was used (Table 2) contamination increased somewhat after a similar period of incubation in penicillin. This difference presumably indicates that bacteria present in non-sterile media enter excised tissues and contribute to the endogenous contamination.

The above data show that by using sterile buffers in conjunction with 15–1000 mg/l. penicillin, bacterial populations can be kept to low levels in experiments of short duration. Furthermore, penicillin in this concentration range, in experiments of similarly short duration, is without effect on leucine or adenine incorporation (Table 4) in either tissue. (In all

TABLE 4. THE EFFECT OF PENICILLIN ON INCORPORATION OF ^{14}C-LEUCINE INTO PROTEIN AND OF ^{14}C-ADENINE INTO RNA

Concn. of penicillin, (mg/l.)	Specific activity			
	Protein		RNA	
	Pea	Cucumber	Pea	Cucumber
0	702	11,820	4·49	19·6
15	710 (101)	—	4·72 (105)	19·6 (100)
100	703 (100)	12,290 (104)	4·39 (98)	20·4 (104)
1,000	—	11,750 (99)	4·52 (101)	—

Segments were pretreated for 1 hr, then 0·5 μc ^{14}C-leucine or ^{14}C-adenine added for 1 hr (pea) or 2 hr (cucumber). Specific activity is expressed as counts/min/mg protein or counts/min/μg RNA. Figures in parenthesis are per cent of control.

incorporation experiments buffers were autoclaved before use.) These findings, in view of the 50- to 100-fold difference in contamination levels between tissues incubated with or without penicillin for 4 hr (Table 3), indicate that bacterial populations of this magnitude within tissues do not contribute to the measured incorporation. (To estimate the numbers of bacteria present per tissue sample in incorporation experiments, the bacterial counts quoted should be divided by five, since each sample of forty 2·5 mm segments weighs about 200 mg.) It is just conceivable that at each concentration tested, penicillin promotes tissue incorporation to the same extent that it depresses bacterial incorporation. However, it is exceedingly unlikely that such a relationship would be maintained at every concentration, for both precursors, and in both tissues. Furthermore cycloheximide, which Whiffen[8] showed to be without effect (at 1000 mg/l.) on the growth of twelve bacterial species, inhibits leucine incorporation by 99 and 98 per cent in pea and cucumber segments respectively (Table 5), at a concentration of 10 mg/l. When tested on pea segments, this concentration of cycloheximide did not diminish the bacterial count of tissue incubated for 18 hr. Thus, although 15 mg/l. penicillin has routinely been used for other incorporation studies in this laboratory,

[8] A. J. WHIFFEN, *J. Bacteriol.* **56**, 283 (1948).

it was considered sound, in order to avoid possible antibiotic interactions, to omit penicillin during subsequent work with streptomycin reported here.

TABLE 5. INHIBITION OF PROTEIN SYNTHESIS BY CYCLOHEXIMIDE

Treatment	Specific activity, counts/min/mg protein	
	Pea	Cucumber
Control	1984	11,470
Cycloheximide, 10 mg/l.	20 (1·0)	210 (1·8)

Segments were pretreated for 1 hr, then 0·5 μc ^{14}C-leucine added for 2 hr. Figures in parenthesis are per cent of control.

From Fig. 1 it is evident that streptomycin in the range 10–100 mg/l. depresses leucine incorporation by both pea and cucumber segments; marked inhibition occurs at 50 and 100 mg/l. Assay of the ethanol-soluble fraction (see Experimental section) revealed that the amount of free ^{14}C-leucine was either unaffected or slightly increased in the presence of streptomycin. Inhibition of leucine incorporation is not, therefore, the result of a reduced rate of uptake. Streptomycin also inhibits adenine incorporation (Table 6), but to a much lesser extent.

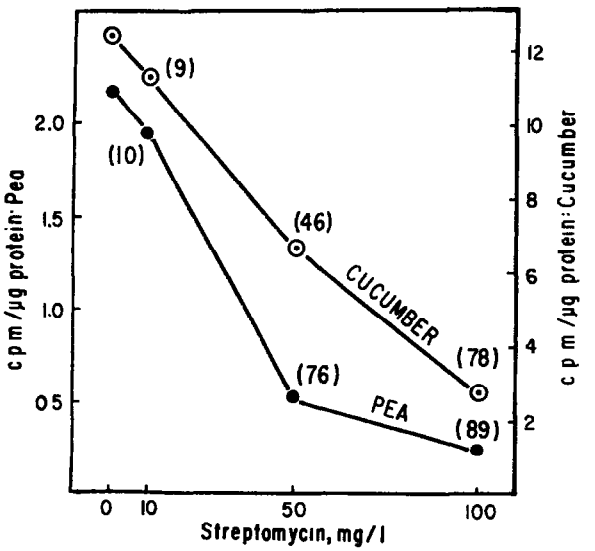

FIG. 1. INHIBITION OF PROTEIN SYNTHESIS BY STREPTOMYCIN.
Segments were pretreated for 2 hr, then 0·5 μc ^{14}C-leucine added for 2 hr. Figures in parenthesis represent the per cent inhibition at each concentration.

To investigate the influence of the antibiotics during prolonged periods of incubation, pea segments were pretreated for 16 hr in the presence of streptomycin or penicillin, and then transferred to fresh medium plus ^{14}C-leucine for 1 hr. At 15 or 100 mg/l., penicillin is still

essentially non-inhibitory (Table 7), while 1000 mg/l. inhibits incorporation by 14 per cent under these conditions. 10 mg/l. streptomycin, which is only slightly inhibitory in experiments of short duration (Fig. 1), now inhibits leucine incorporation by 50 per cent. Compared with freshly-excised tissue, leucine incorporation by pea segments rises 10-fold or more following 16 hr pretreatment. It could therefore be suggested that the inhibitions recorded in Table 7 reflect either the suppression of bacterial growth over this period, or a fundamental change in the characteristics of the protein-synthesizing system during pretreatment, such that incorporation becomes increasingly sensitive to inhibition. However, the fact that lower concentrations

TABLE 6. THE EFFECT OF STREPTOMYCIN ON RNA SYNTHESIS

Concn. of streptomycin (mg/l.)	Specific activity, counts/min/μg RNA	
	Pea	Cucumber
0	9·06	16·1
10	—	16·4 (102)
50	8·40 (93)	14·7 (91)
100	—	13·3 (83)

Segments were pretreated for 2 hr, then 0·5 μc ^{14}C-adenine added for 2 hr. Figures in parenthesis are per cent of control.

TABLE 7. THE EFFECT OF PROLONGED INCUBATION IN PENICILLIN OR STREPTOMYCIN ON PROTEIN SYNTHESIS BY PEA SEGMENTS

Treatment	Counts/min	Mg protein	Specific activity (counts/min/mg protein)	% of control
Buffer	42,485	2·60	16,340	—
Penicillin, 15 mg/l.	41,000	2·60	15,770	97
Penicillin, 100 mg/l.	39,760	2·57	15,470	95
Penicillin, 1,000 mg/l.	37,815	2·68	14,110	86
Streptomycin, 10 mg/l.	20,910	2·55	8200	50

Segments were pretreated for 16 hr, then transferred to fresh medium plus 0·5 μc ^{14}C-leucine for 1 hr.

(15–100 mg/l.) of penicillin over an extended period effect little inhibition of incorporation even though bacterial growth is curbed considerably (Table 3), would seem to discount the first possibility. In addition, it was found that leucine incorporation by pea segments which had been pretreated for 16 hr in buffer (without penicillin) was still highly sensitive to 10 mg/l. cycloheximide (98 per cent inhibition). To examine the second possibility, namely that the enhanced rate of protein synthesis following pretreatment is more sensitive to inhibition, pea segments were pretreated in buffer for 16 hr, then transferred either to fresh buffer or to streptomycin or penicillin for 2 hr. ^{14}C-leucine was then added for 1 hr and incorporation measured. The results of Table 8 demonstrate that under these conditions 1000 mg/l. penicillin is non-inhibitory, while streptomycin at 10 mg/l. inhibits incorporation by 12 per cent. These findings are similar to those obtained in short-term experiments (Table 4 and

Fig. 1) and indicate that in this regard the behaviour of the system towards the antibiotics has not changed. Thus the enhanced influence of these concentrations of penicillin and streptomycin during long treatment periods is presumably simply the result of high levels of antibiotic accumulation. However, this factor is one to be considered in the planning of long-term experiments.

TABLE 8. THE EFFECT OF PENICILLIN AND STREPTOMYCIN ON PROTEIN SYNTHESIS BY PEA SEGMENTS AFTER PRETREATMENT IN BUFFER

Treatment	Specific activity (counts/min/mg protein)	% of control
Buffer	16,700	—
Penicillin, 1,000 mg/l.	17,390	104
Streptomycin, 10 mg/l.	14,730	88

Segments were pretreated in buffer for 16 hr, then transferred either to fresh buffer or to streptomycin or penicillin for 2 hr. Incorporation was then measured by the addition of 0·5 μc ^{14}C-leucine for 1 hr.

Since penicillin reduced the bacterial population of cucumber segments only 10-fold over 18 hr (Table 3), the influence of the bacteriostatic agent chloramphenicol was examined. Chloramphenicol gives somewhat more effective control (Table 9), while the combination with penicillin offers no further advantage. However, even at this relatively low concentration of 50 mg/l., chloramphenicol inhibits leucine incorporation by 9 and 15 per cent in short- and long-duration experiments respectively (Table 10).

TABLE 9. THE EFFECT OF CHLORAMPHENICOL ± PENICILLIN ON BACTERIAL GROWTH IN CUCUMBER SEGMENTS INCUBATED FOR 18 HR IN STERILE BUFFER

Medium	Colonies/g tissue, $\times 10^{-3}$
Buffer	16,000
Chloramphenicol, 50 mg/l.	280
Chloramphenicol, 50 mg/l. + penicillin, 15 mg/l.	280

TABLE 10. INHIBITION OF PROTEIN SYNTHESIS IN CUCUMBER SEGMENTS BY CHLORAMPHENICOL

Treatment	Duration, hr, of: Pretreatment	Duration, hr, of: Incorporation	Specific activity (counts/min/mg protein)	% of control
Buffer	1	2	11,470	
Chloramphenicol, 50 mg/l.	1	2	10,440	91
Buffer	18	1	14,050	
Chloramphenicol, 50 mg/l.	18	1	11,950	85

DISCUSSION

The findings reported here demonstrate that streptomycin strongly inhibits leucine incorporation by pea and cucumber segments and must therefore be considered unsuitable

as an antibiotic for these tissues. Even 10 mg/l. over a prolonged period results in marked inhibition (Table 7). The significance of such inhibition is clearly not limited to studies involving measurement of rates of protein synthesis, but extends to all work in which continued synthesis of protein is essential. For example, induction by auxins of the aspartate-conjugation system of pea segments[9] is abolished by 10–50 mg/l. streptomycin (unpublished results).

It was somewhat surprising to find that even quite high levels of bacterial contamination (up to 10^6 or more per 200 mg tissue) do not contribute to the measured rates of protein and RNA synthesis. Of course, a population of this order distributed throughout the plant tissue will be far less accessible to the supplied isotope than would an equivalent number of bacteria in culture. Even so, it must be concluded that under the conditions studied, bacteria within tissues are unable to compete effectively for exogenously-supplied leucine or adenine. However, this may well not hold for other types of precursor, nor for experiments in which incorporation is studied for periods longer than the 1–2 hr used here. Moreover, it is clearly desirable to reduce contamination as much as possible in order to avoid possible side-effects from products of bacterial metabolism. For this purpose penicillin appears to be quite suitable. At 15–100 mg/l. penicillin is essentially without effect on either leucine or adenine incorporation and the antibiotic gives good control of bacterial growth, except during prolonged incubation of cucumber segments.

EXPERIMENTAL

Plant material. Pea seed (*Pisum sativum* L., var. Alaska) and cucumber seed (*Cucumis sativus* L., var. White Spine) were surface-sterilized in hypochlorite (10 per cent Javex) and germinated in sterilized vermiculite for 7 and 5 days respectively, in a dark growth room at 25°. The apical 1 cm portions of the cucumber hypocotyl and of the third internode of the pea epicotyl were used. These portions were excised as four 2·5 mm segments. Each tissue sample for incorporation studies comprised forty such segments and weighed about 200 mg.

Chemicals. DL-Leucine-1-^{14}C (3·93 mc/mmole) and adenine-8-^{14}C (2·42 mc/m-mole) were purchased from New England Nuclear Corp. Penicillin G (benzylpenicillin, potassium salt) was obtained from Nutritional Biochemical Co., streptomycin sulphate was a product of Eli Lilly and Co., and chloramphenicol was a gift from Parke, Davis and Co. Ltd.

Measurement of protein and RNA synthesis. Following excision, segments were rinsed briefly with distilled water, transferred to the pretreatment medium in Erlenmeyer flasks and shaken on a metabolic shaker at 25°. In experiments of short duration (2–4 hr) the volume was 5 ml and labelled precursor (50 μl, 0·5 μc) was added directly to the flask at the appropriate time. For longer pretreatments, segments were incubated in 10 ml, then transferred, for the incorporation period, to 5 ml of fresh medium supplemented with 0·5 μc of precursor. All solutions were made up in 5 mM maleate buffer, pH 5·5, autoclaved before use.

At the end of the incorporation period, segments were removed, rinsed well with distilled water and plunged immediately into 2 ml of boiling 95% ethanol. They were then homogenized and washed successively with ice-cold 5% trichloroacetic acid and lipid solvents, by a procedure similar to that of Osborne.[10]

For adenine incorporation, the final pellet was hydrolysed in 1·5 ml of 0·3 N KOH for 1 hr. Following acidification of the supernatant with perchloric acid and removal of DNA and potassium perchlorate, the hydrolysate was adjusted to 3 ml with water. RNA was estimated on a diluted aliquot from the absorption at 260 nm, with a small correction for scattering at 320 nm. Yeast RNA (Worthington Biochemical Corp.), hydrolysed under similar conditions, was used as a standard. Duplicate 0·5 ml aliquots were counted by liquid scintillation (Nuclear-Chicago, Mark 1, Model 6860) in 10 ml of a 10:8 scintillator–methyl cellosolve mix. The scintillator fluid contained 4 g 2,5-diphenyloxazole and 50 mg *p-bis*-(*o*-methylstyryl)-benzene per litre of toluene. Efficiency of the system was about 77 per cent.

When leucine incorporation was studied, the pellet after lipid extraction was hydrolysed in 2 ml of 1 N NaOH at 100° for exactly 5 min. One ml of the supernatant was taken for protein estimation by biuret and duplicate 0·4 ml portions were counted by liquid scintillation as described above, but with the addition of 0·1 ml of 98% formic acid to prevent spurious counts from the alkali.[11]

[9] M. A. Venis, *Nature* **202**, 900 (1964).
[10] D. J. Osborne, *Plant Physiol.* **37**, 595 (1962).
[11] T. C. Hall and E. C. Cocking, *Biochem. J.* **96**, 626 (1965).

The reported specific activity figures are the means of duplicate determinations. The spread about the mean was not more than ±10 per cent.

Bacterial counts. Two gramme of segments, freshly-excised or after appropriate incubation, were homogenized in 2 ml of sterile water, the homogenate forced through a cotton wool plug in the barrel of a hypodermic syringe, and the debris-free extract collected in a culture tube. All equipment was sterile and these manipulations were carried out as aseptically as possible. Serial dilutions were made in "Difco" nutrient broth and plated out in "Difco" nutrient agar supplemented with 1% glucose. Colonies were counted after incubation at 30° for 3 days.

Acknowledgements—I wish to thank H. Bork for excellent technical assistance and Dr. E. B. Roslycky of this Institute for valuable advice on bacterial counting techniques.