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The Effect of Penicillin, Novobiocin, Streptomycin and Vancomycin on Membrane Synthesis by Protoplasts of *Bacillus megaterium*

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(Received 29 March 1963)

There are few antibiotics whose mechanism of action is thoroughly understood. For some, it has been suggested that the lethal action is by interference with the synthesis or the function of the cell membrane; but in general the evidence is insufficient to decide whether the membrane is the primary point of attack of the antibiotic.

Penicillin is thought to act by inhibiting the synthesis of the cell wall in susceptible organisms, and the breakdown of the osmotic barrier which has been observed in penicillin-treated bacteria is generally supposed to be a consequence of this inhibition (for review see Salton, 1960). However, Prestidge & Pardee (1957) reported that inhibitors of protein synthesis largely prevented the leakage of cell contents from organisms exposed to penicillin, and concluded that penicillin caused the formation of an enzyme that attacked the cell membrane. Novobiocin was shown by Brock &

Brock (1959) to produce somewhat similar effects in *Escherichia coli*: growing cells treated with the antibiotic lost protein and ribonucleic acid to the medium and suffered an increase in permeability, whereas non-growing organisms were unaffected. It was concluded that novobiocin exerts its bactericidal action by preventing the synthesis of new membrane in growing bacteria. Streptomycin was shown to cause the leakage of nucleotides and amino acids from growing *E. coli* without altering the intracellular concentration of these compounds, and this leakage was attributed to the formation of defective membrane (Anand & Davis, 1960); the hypothesis was supported by the discovery that growth in the presence of streptomycin accelerates the efflux of K^+ ions from the same organism (Dubin & Davis, 1961). Reynolds (1962) has compared the effect of penicillin and vancomycin on *Bacillus megaterium* and *Staphylococcus aureus*. High concentrations of sucrose or salts prevented the inhibition by penicillin of the accumulation of

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^{14}C -labelled amino acids; but the inhibition by vancomycin was unaffected by osmotic stabilizers. Vancomycin caused lysis of *B. megaterium* even in the presence of 10% (w/v) sucrose, and treatment of the organism with penicillin in the same medium led to the production of stable protoplasts.

Although each of these antibiotics eventually impairs the osmotic integrity of sensitive organisms, alternative hypotheses have been proposed for their primary sites of action; the evidence for a direct effect on the cell membrane, although cogent for some of the drugs, is not conclusive for any. Such an effect would be established if it could be shown that the drug specifically inhibited the synthesis of the membrane. There appear to have been no detailed studies of such synthesis, although McQuillen (1956) and Fitz-James (1958) observed in growing protoplasts the formation of enzymes and of phospholipids that were probably located in the membrane. McQuillen (1955) described the incorporation of radioactive compounds into protoplasts of *B. megaterium* but did not estimate incorporation into the membrane fraction. The present paper extends the scope of these investigations, and describes a method of estimating the synthesis of both the protein and the lipid components of the protoplast membrane. The effects of the four antibiotics on this synthesis have been investigated directly; the experiments have involved a comparison of the synthesis of membrane and of other cellular constituents in the presence of concentrations of antibiotic which caused only partial inhibition.

MATERIALS AND METHODS

Radioactive compounds. ^{14}C -labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks., and were used without dilution by carrier; $1\mu\text{C}$ represented $23\mu\text{g.}$ of L- ^{14}C arginine (generally labelled), $20\mu\text{g.}$ of L- ^{14}C histidine, $22\mu\text{g.}$ of L- ^{14}C tyrosine (generally labelled) and $59\mu\text{g.}$ of ^{14}C glycerol.

Maintenance of the organism. *Bacillus megaterium* KM was used. It was subcultured at 30° in tubes of 'C medium' (McQuillen & Roberts, 1954) containing 0.5% of glucose.

Growth conditions. A sample (0.1 ml.) from a 1-day-old culture was added to a 1 l. flask containing 900 ml. of 'C medium' supplemented with 0.1% of glucose. The flask was fitted with a sintered-glass tube through which sterile air was passed. After 16 hr. of growth at 30° , when the culture had reached about 0.1 mg. dry wt./ml., the bacteria were harvested by centrifuging and washed once with 'C medium'.

Preparation of protoplasts. The bacteria were suspended at 10 mg. dry wt./ml. in 'Pr medium', which contained (per litre): sucrose, 100 g.; KCl, 4 g.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 4 g.; NH_4Cl , 2 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 350 mg.; Na_2SO_4 , 150 mg.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg.; adjusted with NaOH to pH 7.0. Then 200 $\mu\text{g.}$ of lysozyme/ml. was added, and the suspen-

sion was incubated at 30° until microscopic examination showed quantitative conversion into protoplasts.

Incubation of protoplasts. Attempts to centrifuge protoplasts and resuspend them in fresh medium resulted in extensive lysis. The procedure therefore adopted for inoculation was to dilute the lysozyme preparation with 9 vol. of 'Pr medium' and to add 1 vol. from this dilute suspension to 9 vol. of incubation medium.

In preliminary experiments, the effect of supplementing 'Pr medium' was investigated. The addition of peptone to the medium caused a greater stimulation of the incorporation of radioactive compounds than did the addition of an amino acid mixture. For the experiments described below, the incubation medium consisted of 'Pr medium' supplemented with 1% (w/v) of glucose and 0.1% of Evans peptone (final concentrations) and readjusted with 2N-NaOH to pH 7.0. Radioactive compounds were added at 0.1 $\mu\text{C/ml.}$ (final concn.).

The mixture (volume 4–9 ml.) was placed in 150 ml. conical flasks which were incubated at 30° . The flasks were fixed to a platform 30 cm. in length, and this was rocked so that its end travelled through 2 cm. at 25 cycles/min. This gentle agitation permitted the protoplasts to be aerated without suffering lysis.

To test for possible incorporation by intact cells, control incubations were carried out with lysed protoplasts: 1 vol. from the thick suspension (10 mg./ml.) of protoplasts was diluted with 9 vol. of water, and the mixture was shaken vigorously; 1 vol. from this lysate was then inoculated into 9 vol. of incubation medium.

Determination of the incorporated radioactivity. (a) Incorporation into trichloroacetic acid-precipitable material. A 1 ml. portion from the incubation mixture was added to 1 ml. of 10% (w/v) trichloroacetic acid in a small tube. The precipitate was collected on a membrane filter (Oxoid; Oxo Ltd., London, E.C. 4), and the tube was washed out twice with 5 ml. of 1% (v/v) acetic acid and once with 2 ml. of water. The washings were successively passed through the filter, and this was then removed and allowed to dry at room temperature. (b) Incorporation into the membrane fraction. A 1 ml. portion from the incubation mixture was added to a flask containing 9 ml. of a solution in water of deoxyribonuclease (1 $\mu\text{g./ml.}$) and carrier (400 $\mu\text{g.}$ of glycerol, arginine or histidine/ml. or, for tyrosine, a saturated solution). Lysis was immediate, and the residue from the lysed protoplasts was collected on a membrane filter. The flask was washed out with 5 ml. of water, the washing was passed through the filter, and this was then removed and allowed to dry.

The dried membrane filter was gummed to a planchet and radioactivity was measured with an end-window Geiger-Müller tube (1 μC gave about 130 000 counts/min.) or with a gas-flow counter (1 μC gave about 610 000 counts/min.). At least 1000 counts were recorded; the rate was corrected for background.

Antibiotics. Penicillin G (potassium salt) was obtained from Squibb and Sons, streptomycin sulphate from Glaxo Ltd., and novobiocin (sodium salt) from The Upjohn Co. Vancomycin (free base) was a generous gift from Eli Lilly and Co.

Enzymes. Crystalline lysozyme was bought from the Armour Pharmaceutical Co., Eastbourne, and crystalline deoxyribonuclease and ribonuclease were from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

EXPERIMENTAL AND RESULTS

Incorporation of radioactive compounds into the membrane

The estimation of incorporation into the membrane involved the filtration of membranes from a lysate of protoplasts. It was therefore necessary to show that these membranes were not contaminated with intracellular particles, especially actively synthesizing ribosomes. Attempts were made to remove any such contaminants by exhaustively washing the filters with water, 1M-sodium chloride or 10% (w/v) trichloroacetic acid. None of these procedures removed any of the radioactivity that had been incorporated into the membranes from amino acids or glycerol. In another experiment, protoplasts were lysed after incubation with [^{14}C]tyrosine; treatment of the lysates with sodium chloride or with ribonuclease failed to decrease the amount of radioactivity found in the membrane fraction.

The radioactive amino acids used in these experiments were chosen from those that would be expected neither to be converted into other amino acids nor to give rise to fatty acids. A series of experiments was carried out to identify the residues into which these amino acids were incorporated in the membrane. Batches (9 ml.) of protoplasts were incubated for 3 hr. in the presence of radioactive arginine, histidine or tyrosine, harvested by centrifuging and lysed by the addition of 10 ml. of water containing 10 μg . of deoxyribonuclease/ml. The membranes were recovered by centrifuging and washed twice with 10 ml. of a solution of carrier (1 mg./ml. or, for tyrosine, a saturated solution). The membrane pellet was hydrolysed with 6N-hydrochloric acid in a sealed tube for 15 hr. at 105°, the acid was removed under reduced pressure, and the hydrolysate was twice moistened and thoroughly dried. An aqueous extract of the residue was applied to Whatman no. 1 paper, and two-dimensional chromatography was carried out as described by McQuillen & Roberts (1954). Radioautograms (Ilford X-ray film) showed that each amino acid had given rise to only one radioactive spot, which was in the position expected for that amino acid (Roberts, Abelson, Cowie, Bolton & Britten, 1955). A similar investigation of the fate of radioactive glycerol showed that this compound had given rise to two spots, one in the position expected for glycerol, the other in the position expected for glycerophosphate.

Incorporation into the trichloroacetic acid-precipitable and membrane fractions

The purpose of the investigations described in the present paper was to determine whether any

of the antibiotics selectively inhibited the synthesis of the protoplast membrane. In each experiment, the incorporation of a radioactive compound into the membrane was compared with its incorporation into the trichloroacetic acid-precipitable fraction. Fig. 1 shows the course of incorporation of radioactive amino acids or glycerol in the absence of antibiotic. The uptake of arginine into the membrane of whole protoplasts was about one-sixth of that into the trichloroacetic acid-precipitable fraction, of histidine about one-eighth and of tyrosine about one-fifth. On the other hand, three-quarters of the incorporated glycerol was found in the membrane. The incorporation into lysed protoplasts was less than 5% of that into whole protoplasts.

Effect of antibiotics

Penicillin. The effect of penicillin on incorporation into protoplasts was measured at concentrations up to 100 μg ./ml. Even at this concentration, the antibiotic caused no inhibition of the incorporation of [^{14}C]glycerol (Fig. 2); similar

Fig. 1. The incorporation of radioactive compounds. Protoplasts (continuous line) or lysed protoplasts (broken line) were incubated with: (a) [^{14}C]arginine; (b) [^{14}C]histidine; (c) [^{14}C]tyrosine; (d) [^{14}C]glycerol. Incorporation was measured in the trichloroacetic acid-precipitable fraction (○) and the membrane fraction (●). Radioactivity was determined with the end-window counter. Experimental details are given in the text.

results were obtained with the amino acids. By contrast, 0.3 $\mu\text{g.}$ of penicillin/ml. completely inhibited the growth of the intact bacteria, as measured turbidimetrically, in 'C medium' supplemented with glucose (1 %, w/v) and peptone (0.1 %).

Novobiocin. The effect of 0.2 $\mu\text{g.}$ of novobiocin/ml. on the incorporation of [^{14}C]histidine into protoplasts is shown in Fig. 3. The antibiotic caused a progressive inhibition of incorporation into the trichloroacetic acid-precipitable fraction; at the end of 3 hr. the total incorporation in the novo-

Fig. 2. Effect of penicillin on the incorporation of [^{14}C]glycerol. Protoplasts (continuous line) were incubated in the absence of the drug (○) or with 100 $\mu\text{g.}$ of penicillin/ml. (●). Lysed protoplasts (broken line) were incubated in the absence of the drug. Incorporation was measured in: (a) the trichloroacetic acid-precipitable fraction; (b) the membrane fraction. Radioactivity was determined with the gas-flow counter. Experimental details are given in the text.

Fig. 3. Effect of novobiocin on the incorporation of [^{14}C]histidine. Protoplasts (continuous line) were incubated in the absence of the drug (○) or with 0.2 $\mu\text{g.}$ of novobiocin/ml. (●). Lysed protoplasts (broken line) were incubated in the absence of the drug. Incorporation was measured in: (a) the trichloroacetic acid-precipitable fraction; (b) the membrane fraction. Radioactivity was determined with the gas-flow counter. Experimental details are given in the text.

biocin-inhibited culture was only 50 % of that in the control. Incorporation into the membrane fraction was affected to the same extent. The presence of novobiocin at lower concentrations caused less inhibition; but, for a given concentration of the drug, the percentage inhibitions of incorporation into the trichloroacetic acid-precipitable fraction and into the membrane fraction were closely similar. The effect of novobiocin on incorporation of the other amino acids showed a similar pattern, but the incorporation of [^{14}C]glycerol was only slightly affected by 0.2 $\mu\text{g.}$ of novobiocin/ml. (Fig. 4).

Streptomycin. The addition of 0.1 $\mu\text{g.}$ of streptomycin sulphate/ml. greatly inhibited the incorporation of [^{14}C]arginine (Fig. 5). After 2 hr. of incubation, incorporation into both the trichloroacetic acid-precipitable and the membrane fractions ceased in the streptomycin-inhibited culture. There was no difference at any time in the extents

Fig. 4. Effect of novobiocin on the incorporation of [^{14}C]glycerol. The conditions were as given for Fig. 3.

Fig. 5. Effect of streptomycin on the incorporation of [^{14}C]arginine. Protoplasts (continuous line) were incubated in the absence of the drug (○) or with 0.1 $\mu\text{g.}$ of streptomycin/ml. (●). Lysed protoplasts (broken line) were incubated in the absence of the drug. Incorporation was measured in: (a) the trichloroacetic acid-precipitable fraction; (b) the membrane fraction. Radioactivity was determined with the end-window counter. Experimental details are given in the text.

Fig. 6. Effect of streptomycin on the incorporation of [^{14}C]glycerol. Protoplasts (continuous line) were incubated in the absence of the drug (○) or with 0.5 μg . of streptomycin/ml. (●). Lysed protoplasts (broken line) were incubated in the absence of the drug. Incorporation was measured in: (a) the trichloroacetic acid-precipitable fraction; (b) the membrane fraction. Radioactivity was determined with the end-window counter. Experimental details are given in the text.

Fig. 7. Effect of vancomycin on the incorporation of [^{14}C]tyrosine. Protoplasts (continuous line) were incubated in the absence of the drug (○) or with 2 μg . of vancomycin/ml. (●). Lysed protoplasts (broken line) were incubated in the absence of the drug. Incorporation was measured in: (a) the trichloroacetic acid-precipitable fraction; (b) the membrane fraction. Radioactivity was determined with the gas-flow counter. Experimental details are given in the text.

to which incorporation into the two fractions were diminished by the antibiotic. Similar experiments were carried out with lower and higher concentrations of streptomycin, and with histidine or tyrosine as the radioactive amino acid. In every case the effect of a given concentration of streptomycin on incorporation into the trichloroacetic acid-precipitable fraction was similar to its effect on incorporation into the membrane fraction. Fig. 6 shows that the

Fig. 8. Effect of vancomycin on the incorporation of [^{14}C]glycerol. The conditions were as given for Fig. 7.

incorporation of [^{14}C]glycerol, unlike that of the amino acids, was affected relatively slightly even by 0.5 μg . of streptomycin sulphate/ml.

Vancomycin. The addition of 2 μg . of vancomycin/ml. caused a decrease in the rate of incorporation of [^{14}C]tyrosine (Fig. 7); at the end of 3 hr. the cumulative inhibition reached 75%. The incorporation of [^{14}C]tyrosine was inhibited less by lower concentrations of vancomycin; at each concentration, the extent of inhibition of incorporation was the same for the trichloroacetic acid-precipitable fraction as for the membrane fraction. Experiments were carried out in which the effect of the antibiotic on the incorporation of [^{14}C]histidine was examined; the results were very similar to those reported in Fig. 7. Vancomycin, unlike novobiocin and streptomycin, inhibited the incorporation of glycerol as strongly as that of amino acids; Fig. 8 shows the effect of 2 μg . of the drug/ml. on the incorporation of [^{14}C]glycerol.

DISCUSSION

Each of the three radioactive amino acids used in these experiments was incorporated into the protein of the protoplast membrane without conversion into other compounds; radioactive glycerol was incorporated into the lipid, also without giving rise to other substances. (Teichoic acid is absent from these membranes; Yudkin, 1962.) This fact enabled the use of these compounds as separate indices of the synthesis of the protein and lipid constituents of the membrane. Since treatment of the membranes with sodium chloride, trichloroacetic acid or ribonuclease failed to remove any of the incorporated radioactivity, there can have been little or no contamination of the membrane fraction by other material. Moreover, lysed protoplasts

incorporated negligible amounts of radioactivity, and, since the method used for lysis would have left whole bacteria intact, the results rule out the possibility of a substantial degree of contamination by bacteria.

One-fifth to one-eighth of the radioactivity incorporated from amino acids was found in the membrane. The protein of the membrane accounts for about 6 % of the total dry weight of the organism, or about one-eighth of the total protein (Yudkin, 1963). It therefore appears that membrane protein and other cellular proteins are synthesized in about the same proportions by protoplasts as by intact bacteria. Similarly, the finding that about three-quarters of the glycerol incorporated by protoplasts is located in the membrane is in agreement with the report by Weibull (1957) that 55–75 % of the lipid of the related *Bacillus M* can be accounted for as membrane lipid.

The mass of amino acid incorporated into the protoplasts cannot be estimated, since the extent of dilution of the radioactive amino acids by the peptone is unknown. However, it is possible to compute the quantity of glycerol incorporated. Fig. 1 shows that after 3 hr. of incubation about 6.5 % of the [^{14}C]glycerol added was found in the membrane. This represents an incorporation into the membrane of some 0.4 μg . of glycerol by the protoplasts in each millilitre of the culture. These protoplasts were formed from 100 μg . dry wt. of whole bacteria.

The protoplast membrane accounts for 7.5 % by weight of *B. megaterium* (Yudkin, 1963). The membrane lipids consist of kephalin, which forms 10 % of the membrane, and neutral lipid, which forms 12.9 % of the membrane, and in both types of lipid C_{15} acids predominate (Yudkin, 1962). It can be calculated that of each 100 μg . of dry bacterial mass about 0.2 μg . is represented by glycerol residues in the membrane. The incorporation shown in Fig. 1 is thus equivalent to an increase of some 200 % in the content of glycerol. Such an increase is far greater than could be accounted for by turnover, and must represent a substantial synthesis of membrane lipid.

Of the antibiotics tested in the present work, penicillin had no effect on the incorporation of radioactive compounds into protoplasts, even when present at 300 times the concentration necessary to inhibit growth of the whole organism. This result cannot be reconciled with the hypothesis that penicillin causes the formation of a membrane-destroying substance (Prestidge & Pardee, 1957). The differences in the behaviour of the drug towards intact bacteria and protoplasts can best be explained by supposing that it acts against a component of bacteria which is absent from protoplasts. This conclusion is in accordance with the widely

accepted theory that penicillin exerts its bactericidal action by blocking cell-wall synthesis in growing bacteria (Lederberg, 1957; Park & Strominger, 1957).

The results of the present experiments provide no direct evidence of the formation of defective membrane in the presence of novobiocin, streptomycin or vancomycin. All three drugs affected incorporation into the membrane fraction to the same extent as that into the trichloroacetic acid-precipitable fraction. However, novobiocin and streptomycin inhibited the incorporation of ^{14}C -labelled amino acids into protoplasts more strongly than that of [^{14}C]glycerol: the membrane formed under these conditions would be disproportionately rich in lipid and might thus be functionally defective. On the other hand, the ratio of lipid to protein within the cytoplasm would also change in the presence of novobiocin or streptomycin, and such an imbalance might equally be detrimental. For this reason, the lethal action of these antibiotics cannot be attributed with certainty to an effect on the membrane.

Davis & Feingold (1962) have pointed out that the accumulation of a wall precursor does not necessarily reflect the site of action of a drug. This warning is pertinent to a discussion of the action of vancomycin which, although it causes the accumulation of hexosamine-containing uridine nucleotides (Reynolds, 1961), exhibits several differences in behaviour from penicillin. The conclusion that the wall is not the primary site of action of vancomycin (Reynolds, 1962) is supported by the results of the present experiments. Vancomycin, however, unlike novobiocin and streptomycin, inhibited the synthesis of protein and of lipid equally, so that there is no evidence that this drug causes the formation of defective membrane by disproportionate synthesis of its two major components.

Shockman & Lampen (1962) have examined the growth of 'protoplasts' of *Streptococcus faecalis* by a turbidimetric technique. Penicillin did not prevent this growth; but several other antibiotics, including novobiocin, streptomycin and vancomycin, inhibited the growth of 'protoplasts' and intact organisms at about the same concentrations. These results provide further evidence that the damaging effect of novobiocin, streptomycin and vancomycin on the cell membrane does not result from an interference with the synthesis of cell wall.

SUMMARY

1. Growing protoplasts of *Bacillus megaterium* KM rapidly incorporated ^{14}C -labelled arginine, histidine, tyrosine and glycerol. A method is described of estimating the radioactivity of the membrane fraction of the protoplasts.

2. Each amino acid was incorporated into the protein of the membrane, and the glycerol into the lipid, without giving rise to other residues.

3. The presence of high concentrations of penicillin had no effect on the rates of incorporation.

4. Both novobiocin and streptomycin inhibited the incorporation of amino acids, and, to a much smaller extent, of glycerol; vancomycin inhibited the incorporation of amino acids and glycerol equally. There was no evidence for a selective inhibition, by any of these antibiotics, of incorporation into the membrane fraction compared with the total cell protein and lipid.

I am indebted to Dr K. McQuillen and Dr B. D. Davis for their interest and encouragement. I thank the Medical Research Council for a Scholarship, and the Commonwealth Fund, New York, for a Harkness Fellowship. This investigation was supported in part by grant G-9078 from the National Science Foundation to Dr B. D. Davis.

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'Glucose 6-Phosphate-Dehydrogenase' Activity and Thiol Content of Thymus Nuclei from Control and X-Irradiated Rats

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(Received 22 March 1963)

Creasey & Stocken (1959) showed that the formation of acid-labile phosphate by nuclei isolated from rat thymus gland was completely prevented by exposing the animals to a 100 r. dose of X-rays. The possible function of 'glucose 6-phosphate dehydrogenase' (EC 1.1.1.49) in this nuclear phosphorylation (Ord & Stocken, 1962a) led to an examination of the activity of the enzyme after irradiation. The results suggested that alterations in the concentration of thiol (SH) compounds in the nuclei from normal rats might influence the activity of the dehydrogenase and that such alterations might be produced by X-irradiation. Glutathione and glutathione reductase (EC 1.6.4.2) have been described in nuclei from calf thymus (Stern & Timonen, 1954; Wang, 1962), and a study of nuclear thiol groups has been made by Klouwen (1962). The present paper indicates how the method

of preparation of the nuclei affects the nature and amount of SH compounds found. The interactions between 'glucose 6-phosphate dehydrogenase' and glutathione reductase have also been examined and shown to complicate the analysis of the effects of X-irradiation on the nuclei.

METHODS

Animals. These were taken from the laboratory strain of Wistar rats. Whenever possible we used females that were put from the time of weaning into a room with a 12 hr. light period, from 8 a.m. to 8 p.m. In all cases the animals were of 100–140 g. body weight (4–6 weeks old).

Nuclei. These were prepared as described by Ord & Stocken (1961) in either 'ionic medium' [KCl (85 mm), NaCl (8.5 mm), CaCl₂ (2.5 mm), MgCl₂ (2.5 mm) and triethanolamine hydrochloride (5 mm), adjusted to pH 7.2] or in 'sucrose medium' [sucrose (0.25 M), CaCl₂ (5 mm) and