EVIDENCE FOR THE IMPORTANCE OF PUROMYCYL PEPTIDES IN THE INHIBITION BY PUROMYCIN OF CELL AGGREGATION IN VITRO

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SUMMARY

Trypsin-dissociated cells from the muscle tissue of 9-day-old chick embryos were employed to investigate the effects of cycloheximide and a puromycin-cycloheximide mixture on cell aggregation, protein synthesis and respiratory metabolism.

Cycloheximide when introduced at a concentration of 10 µg/ml into a suspension of cells in Eagle's MEM inhibited aggregation by 25% at 24 h. At this time an inhibition of 40% was apparent in the presence of a mixture of cycloheximide and puromycin both at a concentration of 10 µg/ml.

Both cycloheximide and the cycloheximide-puromycin mixture arrested protein synthesis of rotated cells by 90% within 15 min of introducing the antibiotics into cell suspensions. The antibiotics retained their inhibitory effects on protein synthesis for the 24-h period of rotation.

Cycloheximide inhibited cellular oxygen uptake and carbon dioxide evolution of rotated cells by 25% at the end of the 24-h experimental period. At this time an inhibition of 30% was observed in the presence of the cycloheximide-puromycin mixture. The release of radioactive carbon dioxide by cycloheximide-treated cells was inhibited by 46% at 24 h. In the presence of the antibiotic mixture, 14CO2 release was inhibited by 30% at 4 h, but after 8 h very little further 14CO2 was evolved.

As a control, puromycin (10 µg/ml) inhibited cell aggregation and respiration to an extent similar to that previously reported.

The results are discussed in terms of puromycyl peptides producing a metabolic effect on cell aggregation. It is considered that this is additional to the effect of puromycin inhibiting aggregation through the arrest of protein synthesis.

INTRODUCTION

Moscona & Moscona (1963, 1966) showed that the aggregation of trypsin-dissociated embryonic chick retinal cells was inhibited by puromycin. They concluded that owing to the supposed specificity (Nathans & Neidle, 1963) of this antibiotic as a binding inhibitor of protein synthesis (Yarmolinsky & de la Haba, 1959), cell aggregation was dependent on protein synthesis. However, from studies on the aggregation of embryo chick fibroblasts in the presence of puromycin, Kemp, Jones, Cunningham & James (1967) reasoned that arrested protein synthesis could not adequately explain the observed pattern of inhibited aggregation. They proposed an alternative explanation that puromycin might interfere with the progress of aggregation by adversely affecting cellular metabolism. Dunn, Owen & Kemp (1970) established that puromycin depressed the carbohydrate metabolism of trypsin-dissociated fibroblastic cells in addition to blocking protein synthesis. The pattern of inhibition of
aggregation was considered to be consistent with the gradual depression of metabo-
lism rather than the rapid effect on protein synthesis.

Puromycin is known to act on protein synthesis by attaching to the carboxyl-
terminal end of the peptide chain and stopping addition of further amino acids
present on ribosomes is normally transferred to the amino group of the next amino-
acyl-tRNA, but in the presence of the antibiotic the carboxyl-activated peptide is
attached to puromycin thus ending the sequential extension of the polypeptide chain
(Nathans, 1964). The nascent protein bound to a puromycin residue (puromycyl
peptide) is then released from the ribosome (Allen & Zamecnik, 1962; Smith, Traut,
Blackburn & Monro, 1965). Gambetti, Gonatas & Flexner (1968) and Edmonds &
Longnecker (1969) have ascribed changes in the fine structure of puromycin-treated
mouse entorhinal cortex cells and rat gastric chief and pancreatic acinar cells to the
action of puromycin peptides. Dunn et al. (1970) suggested that changes in cellular
organelles and, more particularly, mitochondrial damage elicited by puromycyl pep-
tides, might be responsible for the inhibition of carbohydrate metabolism in trypsin-
dissociated cells. Jones & Banks (1969) came to a similar conclusion concerning the
severe inhibition of respiration in slices of cerebral cortex treated with puromycin.

Cycloheximide, a glutarimide antibiotic, has been claimed to be a more specific
inhibitor of protein synthesis than puromycin (Sisler & Siegel, 1967). A comparison
of its effects on cellular carbohydrate metabolism and protein synthesis with those of
puromycin should, then, indicate which of these 2 factors are important in cell aggrega-
tion. Cycloheximide acts on protein synthesis by preventing the initiation of new
peptide chains on ribosomes and the elongation of existing nascent chains (Lin,
Mostello & Hardesty, 1966). Owing to the nature of its inhibitory action on protein
synthesis, cycloheximide retards the puromycin-induced release of puromycyl pep-
tides from ribosomes (Colombo, Felicetti & Baglioni, 1965; Soeiro, Vaughan &
Darnell, 1968). Thus, if the severe disturbance of carbohydrate metabolism and
aggregation observed in trypsin-dissociated muscle cells is due to the toxic effects of
puromycin peptides as suggested by Dunn et al. (1970), cells exposed to a mixture of
cycloheximide and puromycin should manifest less deleterious effects on these
cellular functions.

The aims of this investigation have therefore been to study the effects of cyclo-
heximide and a non-stoichiometric mixture of puromycin and cycloheximide on the
protein synthesis, respiratory metabolism and aggregation of trypsin-dissociated
muscle cells. By comparing these results with those of puromycin-treated controls, it
was hoped it would be possible to determine the importance of puromycyl peptides in
the inhibition of aggregation by puromycin.

MATERIALS AND METHODS

Dissociation

Cells were obtained from skeletal muscle of chick embryos at the thirty-fifth stage of de-
velopment (Hamburger & Hamilton, 1951), equivalent to the ninth day of incubation. The
tissue was dissociated into separate cells using 0.05% (w/v) crude trypsin (Burroughs Wellcome
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1:300) in Hanks's balanced salts solution (BSS) for 10 min at 37 °C (Kemp et al. 1967). After washing in Hanks's BSS, the dissociated cells were resuspended in Eagle's minimum essential medium (MEM) at a concentration of $2 \times 10^6$ cells/ml. Sterile conditions were employed throughout the dissociation and subsequent aggregation procedures.

Aggregation

Aggregation was estimated using the gyratory shaker technique (Moscona, 1961). Aliquots, 3 ml, of the cell suspension were placed in 25-ml Erlenmeyer flasks. The pH of the medium was adjusted to 7.6 by gassing the flasks with 5 % CO2 in air (Paul, 1965). Cycloheximide or puromycin (10 μg/ml) or a mixture of cycloheximide (10 μg/ml) and puromycin (10 μg/ml) were added to cell suspensions, the addition of chemicals being omitted in control suspensions. The flasks were rotated at 70 rev/min on a gyratory shaker for periods of up to 24 h at 37 °C (Kemp, 1970).

Progress in aggregation was estimated by counting the single cells that remained in the cell suspension at set time intervals using a modified Fuchs-Rosenthal haemocytometer. Aggregation was also assessed by microscopical examination of the size, number and structure of the aggregates. Lissamine green (2 % (w/v), aqueous) was used to give a rapid test of cell viability (Kemp et al. 1967).

Manometric techniques

The oxygen uptake of cells in the presence of antibiotics compared with controls was measured on a Gilson Differential Respirometer (Gilson Medical Electronics, Inc.) which gives a more efficient estimate of gaseous exchange than does the conventional Warburg apparatus (Gilson, 1963). The Warburg flasks contained 3 ml of the cell suspension at a concentration of $2 \times 10^6$ cells/ml and were equilibrated with the atmosphere for 15 min before oxygen consumption was measured (Umbreit, Burris & Stauffer, 1957). Readings were taken every 15 min over the course of a 2-h period. The carbon dioxide liberated was absorbed in 0.2 ml of a 20 % (w/v) solution of KOH placed in the centre well.

Carbon dioxide evolution by the cells was measured by the 'direct method' (Umbreit et al. 1957) in which were employed pairs of flasks whose contents were respiring under the same conditions. In one flask the carbon dioxide was absorbed, whereas in the other it was not. Thus in one flask oxygen uptake alone was measured, whereas in the other the result of oxygen uptake and carbon dioxide evolution was measured. It was thereby possible to calculate the amount of carbon dioxide released.

Radioisotope studies

The effect of antibiotics on cellular protein synthesis was estimated from the incorporation of L-[α-14C]leucine (specific activity 261 μCi/mg) into cellular proteins. At predetermined times during the 24-h experimental period, 0.1 ml of a 10 μCi/ml solution of isotopic leucine was added to 3-ml aliquots of the cell suspension. After rotation for a further 15 min, the cells and aggregates were harvested and the proteins were isolated, purified and plated as previously described (Kemp et al. 1967). The samples were counted in a Nuclear Chicago Model 4334 gas-flow (2 % propane:98 % argon) radioactive counter to a standard error of 0.5 % and corrected for background.

The effect of antibiotics on the release of 14CO2 during the process of aggregation was estimated by a modified form (Dunn et al. 1970) of a method developed by Snyder & Godfrey (1961). At the start of the experiment, 1-0 μCi of D-[U-14C]glucose (specific activity 167 μCi/mg) was introduced into each flask. The experiment was terminated at predetermined times by injecting perchloric acid into the flask to give a final concentration of 0.3 M. The carbon dioxide so released was absorbed over a 3-h period in 1 ml of hydroxide of Hyamine-10X (Rohm & Hass, Inc.). The Hyamine was then transferred to a vial containing 15 ml of toluene-based liquid scintillant (Fluoralloy dry mix TLA, Beckman Instruments Ltd.) and counted to 1 % error in a Beckman LS 100 spectrometer.
Fig. 1. Effect of antibiotics (10 μg/ml) on the progressive reduction in the number of single cells remaining in a suspension of trypsin-dissociated embryonic chick muscle cells rotated in Eagle's MEM at 37 °C; a, control; suspensions treated with b, cycloheximide, c, cycloheximide + puromycin, and d, puromycin.

Analytical methods

Protein was estimated using the Folin-Ciocalteu reaction (Lowry, Rosebrough, Farr & Randall, 1951). Total protein was estimated after alkaline hydrolysis with 1 N NaOH for 10 min at 100 °C.

RESULTS

Effect of antibiotics on cell aggregation

It will be seen from Fig. 1 that when compared with the untreated control, 10 μg/ml cycloheximide and a mixture of equal parts of cycloheximide and puromycin both inhibited progress in aggregation, though to a lesser extent than the puromycin-treated specimen. Judging by single-cell counts, the effect was evident within 2 h of the start of the experiment and continued to the end of the 24-h period. A difference in the level of inhibition was manifest at 2 hours and persisted to the end of the experimental period. At this time cell suspensions incubated with cycloheximide contained 25% more free cells than controls rotated in Eagle's MEM alone. A mixture of
Puroniycin inhibition of cell aggregation

Table 1. Effect of antibiotics introduced at 0 h on the incorporation of L-[2-14C]leucine into the proteins of trypsin-dissociated muscle cells rotated in Eagle's MEM

<table>
<thead>
<tr>
<th>Time from start of experiment (h)</th>
<th>Incorporation of L-[2-14C]leucine into TCA-precipitate, cpm/mg protein</th>
<th>Inhibition of protein synthesis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>0</td>
<td>1831</td>
<td>204</td>
</tr>
<tr>
<td>4</td>
<td>1890</td>
<td>179</td>
</tr>
<tr>
<td>8</td>
<td>1649</td>
<td>149</td>
</tr>
<tr>
<td>24</td>
<td>1608</td>
<td>134</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0015</td>
<td>1670</td>
</tr>
<tr>
<td>4</td>
<td>0415</td>
<td>1784</td>
</tr>
<tr>
<td>24</td>
<td>2415</td>
<td>1575</td>
</tr>
<tr>
<td>Puromycin + cycloheximide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0015</td>
<td>1670</td>
</tr>
<tr>
<td>4</td>
<td>0415</td>
<td>1784</td>
</tr>
<tr>
<td>24</td>
<td>2415</td>
<td>1575</td>
</tr>
</tbody>
</table>

* Results represent averages of 5 experiments. TCA, trichloroacetic acid.

cycloheximide and puromycin inhibited cell aggregation by 40% at 24 h compared with 80% in puromycin-containing suspensions. These results were qualitatively reproducible.

Visual examination at 24 h showed that aggregates formed by cells treated with the antibiotics (Figs. 6, 7) were considerably smaller and less compact than in the controls (Fig. 5). In addition, there were considerably fewer aggregates in the suspensions incubated in the presence of cycloheximide and puromycin than in those containing cycloheximide alone.

At 24 h cellular viability had declined by 3% to 95% in the controls but had decreased to 90% in suspensions containing antibiotics.

Effect of antibiotics on cellular protein synthesis

When cycloheximide was introduced at 0 h into a cell suspension to give a final concentration of 10 μg/ml, it arrested incorporation of L-[2-14C]leucine into cellular proteins by nearly 90% within 15 min (Table 1). The antibiotic retained its inhibitory effect on protein synthesis for the 24-h experimental period.

A mixture of cycloheximide and puromycin, both at a concentration of 10 μg/ml, was no less effective in arresting protein synthesis than cycloheximide alone. Within 15 min of introducing the mixture, incorporation of isotopic leucine into cellular proteins was reduced by 92% (Table 1). At the 24-h stage protein synthesis was still inhibited by more than 90%.

Effect of antibiotics on cellular respiration

The uptake of oxygen by trypsin-dissociated cells suspended in Eagle's MEM was markedly inhibited by 10 μg/ml cycloheximide. The effect was apparent at 2 h and
by the 4-h stage had resulted in a 15% reduction in oxygen consumption (Fig. 2). At 10 h inhibition had increased to 25% but it stayed at this level for the remainder of the 24-h experimental period.

In the presence of a mixture of cycloheximide and puromycin both at a concentration of 10 μg/ml, respiration was inhibited to a greater extent than in the presence of cycloheximide alone (Fig. 2). By the 10-h stage oxygen uptake was arrested by 30%, this degree of inhibition being maintained to the end of the experimental period (Table 2).

The effect of the antibiotics on carbon dioxide evolution corresponded to their effect on oxygen uptake (Fig. 3). Cycloheximide decreased cellular carbon dioxide release by 25% at 24 h (Table 2). The presence of puromycin in suspensions containing cycloheximide increased the degree of inhibition at 24 h to 33% (Table 2).
Table 2. Effects of antibiotics (10 μg/ml) on rates of oxygen uptake and carbon dioxide evolution of cells after rotation for 24 h on the gyratory shaker

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of experiments</th>
<th>( \Delta O_2 ) uptake</th>
<th>Inhibition, %</th>
<th>( \Delta CO_2 ) evolution</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>2.0</td>
<td>-</td>
<td>2.4</td>
<td>-</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>8</td>
<td>1.5</td>
<td>25</td>
<td>1.8</td>
<td>25</td>
</tr>
<tr>
<td>Cycloheximide + puromycin</td>
<td>8</td>
<td>1.4</td>
<td>30</td>
<td>1.6</td>
<td>33</td>
</tr>
<tr>
<td>Puromycin</td>
<td>8</td>
<td>0.6</td>
<td>70</td>
<td>0.8</td>
<td>67</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of antibiotics on the release of \(^{14}CO_2\) by trypsin-dissociated embryonic chick muscle cells suspended in Eagle’s MEM containing 1.0 μCi of \( \alpha [U-^{14}C] \) glucose/5 x 10⁶ cells. Curves represent the average of a series of 5 experiments for control (a), and preparations treated with cycloheximide (b), cycloheximide + puromycin (c), and puromycin (d).
It should be noted that cell suspensions incubated with puromycin showed consistently greater inhibition of both oxygen uptake (Fig. 2) and carbon dioxide evolution (Fig. 3) than did those subjected to other treatments. This deleterious effect amounted to approximately 50% at 10 h (Figs. 2, 3), increasing to 70% at 24 h (Table 2).

Studies on the release of radioactive carbon dioxide from cells rotated in the presence of d-[U-14C]glucose were not wholly consonant with the effect of the antibiotics on cellular respiration measured by manometry. The presence of cycloheximide in the suspension medium resulted in a 46% inhibition of 14CO2 evolution by the cells at 24 h (Fig. 4). With a mixture of cycloheximide and puromycin, cellular 14CO2 release was decreased to a greater extent than with cycloheximide alone (Fig. 4). At 4 h a 30% depression of 14CO2 release was manifest. However, little 14CO2 was released by the treated cells after the 8-h stage and by 24 h an inhibition of 80% was evident, a result similar to that shown by puromycin-treated cells (Fig. 4).

DISCUSSION

The present findings show that cycloheximide, which has been claimed to be a specific inhibitor of protein synthesis (Sigler & Siegel, 1967), inhibited cellular respiration by 25% after rotation for 24 h as well as rapidly arresting protein synthesis by 90%. Similar slight inhibitory effects of the antibiotic on respiration have been reported in cultured mammalian cells (Arnow, Brindle, Giuffre & Perlman, 1963), Chlorella (Morris, 1966) and guinea pig cerebral cortex (Jones & Banks, 1969). These authors considered that the detrimental effects of cycloheximide on cellular respiration could be ascribed to the inhibition of protein synthesis resulting in either a general depression of enzyme levels or a reduction of specific enzymes possessing a high turnover rate. Puromycin has been shown previously to be a potent inhibitor of protein synthesis in trypsin-dissociated cells (Kemp et al. 1967; Dunn et al. 1970), but was also found to exert a severe effect on respiration resulting in 70% inhibition at 24 h (Table 2; see also Dunn et al. 1970). If the reduced respiration observed in the presence of cycloheximide can be explained as an indirect consequence of inhibited protein synthesis, it is apparent that a further mechanism must be invoked to explain the additional effects of puromycin.

It has been shown conclusively that the termination of protein synthesis by puromycin results in the release of nascent peptides from ribosomes (Morris & Schweeet, 1961; Morris et al. 1963; Nathans, 1964; Hultin, 1966). Allen & Zamecnik (1962) demonstrated that one puromycin residue is bound to each released peptide. Gambetti et al. (1968) considered that puromycyl peptides were responsible for the disruption in fine structure found on treatment with puromycin in many cell types (Longnecker & Farber, 1967; Edmonds & Longnecker, 1969; Gambetti et al. 1968; Weinstock, 1970). It has been further suggested that changes in cellular fine structure and, in particular, mitochondrial damage could adversely influence respiratory metabolism (Jones & Banks, 1969; Dunn et al. 1970).

Owing to the nature of its effects on protein synthesis, cycloheximide retards the
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release of puromycin (Colombo et al. 1965; Soeiro et al. 1968). Thus, if the severe disturbance of respiratory metabolism observed in the presence of puromycin is due to the toxic effects of puromycin, cells exposed to a mixture of cycloheximide and puromycin should exhibit less deleterious effects on respiration. This reasoning was substantiated by the present results showing that when trypsin-dissociated cells were rotated in the presence of a mixture of cycloheximide and puromycin, both at a concentration of 10 μg/ml, the observed effects on respiration were less severe than those observed in the presence of puromycin alone. The antibiotic mixture inhibited respiration by 30% at the 24-h stage compared with 70% in the presence of puromycin alone. This partial reversal by cycloheximide of the inhibitory action of puromycin on respiratory metabolism occurred despite the fact that the antibiotic mixture was as potent an inhibitor of protein synthesis as either antibiotic alone.

If the present metabolic findings using the antibiotic mixture are indeed due to the suppression by cycloheximide of the release of puromycin, then they reinforce the suggestion of Dunn et al. (1970) that puromycin exerts at least part of its adverse effect on respiration through the toxicity of puromycin. The mechanism of action of this group of compounds on cellular ultrastructure is not known but it seems fairly certain that any disruption in mitochondrial morphology would be reflected in an effect on the respiratory and energy-producing systems which are largely localized in these organelles. The dramatic effects of puromycin on the nucleotide levels of trypsin-dissociated cells (Dunn et al. 1970) are indicative of severe disruption of energy metabolism.

In confirmation of results described by Dunn et al. (1970), Fig. 4 showed that by 8 h puromycin had abolished 14CO2 release by cells rotated in the presence of D-[U-14C]glucose. In the present investigation it was demonstrated that cycloheximide arrested the evolution of 14CO2 by 46% at 24 h, but in the presence of cycloheximide-puromycin mixture, virtually no 14CO2 was released after 8 h. It therefore appears that cycloheximide did not reverse the detrimental effects of puromycin on release of 14CO2. Dunn et al. (1970) explained their findings by postulating that the active uptake of extracellular glucose was highly susceptible to impaired energy production. Isotopic CO2 can only be derived from exogenous glucose and would therefore be more susceptible to impaired glucose uptake than unlabelled CO2 which can be produced from a variety of substrates. Indirect evidence for the susceptibility of glucose uptake, and thereby 14CO2 release, to impaired cellular energy production can be derived from the present finding that cycloheximide, although inhibiting total carbon dioxide evolution by only 25% at 24 h, arrested 14CO2 release by 46% at this time. However, a mixture of cycloheximide and puromycin only reduced the evolution by cells of unlabelled CO2 by 33% at 24 h, which would not seem sufficient completely to arrest glucose uptake. As has been discussed previously (Dunn et al. 1970), it is possible that puromycin could block glucose uptake at the plasma membrane and therefore that its presence in the mixture of antibiotics severely limited the production of 14CO2 from isotopic glucose.

It may well be coincidental that the levels at which aggregation was inhibited by
the antibiotics closely approximated to those at which they exerted their adverse effect on respiratory metabolism. However it is remarkable that cycloheximide, which had only a slight effect on carbohydrate metabolism, inhibited aggregation by 25% at 24 h whereas puromycin caused a drastic reduction in metabolism and diminished aggregation by 80% (Fig. 1; see also Dunn et al. 1970). Since both antibiotics arrested protein synthesis by more than 90%, it is evident that their quantitatively different effects on aggregation cannot be solely attributed to their arresting protein synthesis. It is acknowledged that the aggregation-inhibitory effect of cycloheximide may be due to arrest of protein synthesis. Even in this case, however, one cannot be certain whether this is due to it limiting specific proteins necessary for aggregation or to it restricting the production of rate-limiting enzymes with a fast turnover.

It is suggested that the additional effect of puromycin on cell aggregation is derived from a disruption of energy metabolism, possibly owing to the toxicity of puromycyl peptides. Evidence that these compounds may be responsible for the additional effect can be inferred from the fact that cycloheximide, which prevents the release of puromycyl peptides, reduced the inhibition of puromycin on aggregation from 80 to 40%. It is known that inhibitors of glycolysis and the Kreb’s cycle will reduce aggregation (M. J. Dunn & R. B. Kemp, in preparation) although one cannot be sure that this change is not due to an indirect effect on energy-requiring biosynthetic processes. In the case of puromycin it is certain that the postulated antimetabolite role for puromycyl peptides cannot include an indirect effect on protein synthesis because this is already arrested.

While it is probably true that protein synthesis is necessary for ‘normal’ aggregation, the present results strongly suggest that an additional requirement may well be cellular respiratory metabolism for energy production. It would appear that this requirement does not solely manifest itself on aggregation as an effect on protein synthesis.

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REFERENCES


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Fig. 5. Relatively large aggregates produced by trypsin-dissociated cells at the end of a 24-h period of rotation in Eagle’s MEM at 37 °C. × 90.

Fig. 6. Aggregates formed in 24 h by trypsin-dissociated cells in Eagle’s MEM at 37 °C after cycloheximide had been introduced at 0 h to give a final concentration of 10 μg/2 × 10⁶ cells/ml Eagle’s MEM. Note that these aggregates are smaller than those formed in control preparations (Fig. 5). × 90.

Fig. 7. Aggregates produced by trypsin-dissociated cells rotated for 24 h at 37 °C in Eagle’s MEM containing cycloheximide and puromycin, both at a concentration of 10 μg/2 × 10⁶ cells/ml Eagle’s MEM. × 90.
Puromycin inhibition of cell aggregation