The effects of inhibition of protein glycosylation on the aggregation of *Dictyostelium discoideum*

By CHARLES JOHN MCDONALD\(^1\) AND JEFFREY SAMPSON\(^2\)

*From the Department of Biochemistry, University of Leicester*

**SUMMARY**

At concentrations greater than 10\(\mu g\) ml\(^{-1}\) tunicamycin inhibited the incorporation of \(^{3}H\)mannose into glycoproteins during the early phase of development in *Dictyostelium discoideum*, however, total protein synthesis was unaffected. Tunicamycin also interfered with the normal process of aggregation. In its presence small aggregates were observed at the time of normal aggregation, but amoebae failed to aggregate completely and subsequent development was inhibited. Inhibition of normal aggregation by tunicamycin was found to be reversible. The appearance of cell-associated and secreted cyclic AMP phosphodiesterase and cell-surface contact sites A was prevented by tunicamycin but cell surface cyclic AMP receptor activity developed normally in its presence. Tunicamycin also prevented amoebae from acquiring the ability to chemotact toward cyclic AMP. Addition of exogenous cyclic AMP phosphodiesterase restored the ability of amoebae to chemotact toward cyclic AMP in the presence of tunicamycin. Our data suggest that the primary block in aggregation caused by tunicamycin results from the inhibition of expression of active cyclic AMP phosphodiesterase.

**ABBREVIATIONS**

- cyclic AMP: adenosine 3' 5' cyclic monophosphate
- dimethyl POPOP: 1,4-bis-[\(2\)-(4-Methyl-5-phenyloxazolyl)-3]-Benzene
- DMSO: Dimethylsulphoxide
- i.u.: International Unit of Enzyme Activity
- NP 40: The non-ionic detergent Nonidet P40
- PP0\(^{\prime}\): 2,5-diphenyloxazole
- SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
- TCA: trichloroacetic acid
- TM: Tunicamycin
- \(t_x\): time in hours (x) after the onset of development

**Enzymes quoted**

- PDE: cyclic AMP phosphodiesterase [E.C.3.1.4.17]

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\(^1\)Author's address: Department of Biochemistry, University of Leeds, Leeds, U.K.

\(^2\)Author's address: Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, U.K.
INTRODUCTION

The molecular basis of cellular communication in embryonic development in higher eukaryotes has been difficult to study because of the close packing and adhesion of the cells comprising the tissue. Aggregation of the cellular slime mould Dictyostelium discoideum, however, starts from a field of apparently identical amoebae (Bonner, 1944) and cell movements continue until the amoebae are associated into multicellular aggregates (Bonner, 1949 and 1963). It therefore offers an attractive system for the study of intercellular communication. The molecular basis of aggregation is now well understood (Loomis, 1975; Robertson & Grutsch, 1981) and the possibility has been raised that this system may be responsible for cellular communication and movement during the later multicellular stages of the organism's development (Newell, 1978). Indeed, it has been a subject of conjecture that the mode of D. discoideum aggregation may be found among the as yet uncharacterized cellular communication systems of higher eukaryotes (Robertson & Grutsch, 1981; Gingle, 1977; Guillermet & Mandaron, 1980).

Aggregation of D. discoideum is initiated by certain amoebae secreting pulses of cyclic AMP and the surrounding amoebae responding by positive chemotaxis and the secretion themselves of cyclic AMP pulses (Konijn, 1972). This signal relay system entrains amoebae over a large area into each aggregation centre into which cells move in synchronous waves or spirals (Gerisch, 1968; Alcantara & Monk, 1974; Newell, 1978). The enzymes which control the cyclic AMP signal are developmentally regulated. Prior to aggregation, adenyl cyclase activity is present (Klein & Darmon, 1977; Darmon & Klein, 1978) and cyclic AMP phosphodiesterase and its inhibitor protein are secreted by the amoebae (Pannbacker & Bravard, 1972; Gerisch et al. 1972). Parallel with the acquisition of the ability to chemotact toward cyclic AMP pulses at the onset of aggregation there is a rapid increase in cell surface cyclic AMP phosphodiesterase (Malchow et al. 1972). As aggregation proceeds the synchronous moving waves of amoebae break up into 'streams' (Bonner, 1971), stream formation being mediated through end-to-end cell contact via cell surface contact sites A which also increase in activity during this period (Beug et al. 1973). There is evidence that the cell surface aggregation functions (cyclic AMP receptors, cyclic AMP phosphodiesterase and contact sites A) are synthesized de novo during aggregation (Sampson et al. 1978) as are a large number of secreted enzymes which increase in activity during early development (Loomis, 1975).

Cell surface cyclic AMP phosphodiesterase (Eittle & Gerisch, 1977), the secreted form of the enzyme (Orlow et al. 1981), its inhibitor (Franke & Kessin, 1981) and contact sites A (Muller & Gerisch, 1978) are all glycoproteins as are many membrane components which change during development (Toda et al. 1980). In order to study the role of de novo protein glycosylation during aggregation in D. discoideum we have used the antibiotic tunicamycin which inhibits the
first step in the glycosylation of proteins via N-asparaginyl links (Takatsuki et al. 1971; Tkacz & Lampen, 1975). We report that inhibition of protein glycosylation by tunicamycin (TM) prevented the appearance of cyclic AMP phosphodiesterase and contact sites A but that cell surface cyclic AMP receptor activity developed normally. TM caused a reversible block in development but small aggregates did form in the presence of TM at the normal time of aggregation. TM prevented the development of the ability of amoebae to chemotact toward cyclic AMP but the addition of cyclic AMP phosphodiesterase to TM-inhibited cells restored this activity. The implications of these results are discussed.

**MATERIALS AND METHODS**

**Chemicals**

Tunicamycin was the kind gift of Drs Harper and Herrman, Biotechnology Department, Glaxo Research Limited, Greenford, Middlesex, England. It was stored either desiccated as a powder or as a 1–5 μg ml⁻¹ solution in DMSO at -20°C. DMSO at concentrations up to 0.44 M had no observed effect on development of Dictyostelium or on any of the cellular or biochemical functions measured in this study. Radiochemicals were obtained from Radiochemicals International, Amersham, England. All other chemicals and reagents were of the highest purity available.

**Growth and development of the organism**

Dictyostelium discoideum strain AX2 spores (a kind gift from Dr M. North) were stored on silica gel at 4°C. Germination and growth were carried out in aerated suspensions of HL5 growth medium (1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 20 mM-phosphate buffer, pH 6.9) containing 86 mM-glucose (Watts & Ashworth, 1970). The media contained 200 μg ml⁻¹ streptomycin and 5 μg ml⁻¹ tetracycline. Flasks, at least four times the volume of suspension, were shaken at 175 r.p.m. on a New Brunswick G2 gyratory shaker. Amoebae were harvested during log phase (2–4 x 10⁶ cells ml⁻¹) by centrifugation at 800 g for 10 min at 22°C, washed three times in KK₂ buffer (20 mm-potassium phosphate, pH 6.2) containing 0.5 mm-CaCl₂ and 2 mm-MgSO₄, and finally resuspended in this same buffer at a concentration of 1 x 10⁷ ml⁻¹. Washed amoebae were shaken at 125 r.p.m. and 22°C in chromic acid washed, siliconized flasks and the onset of development (t₀) was measured from the time of final resuspension of the washed amoebae. To observe aggregation, washed amoebae were plated onto 1-2 % agarose in KK₂ buffer at a density of 5 x 10⁵ amoebae cm⁻¹. 55 mm tissue culture dishes were used, each containing 3 ml agarose. Photomicrography was performed using a Wild dissecting microscope fitted with a Nikon cameraback.

**Measurement of chemotaxis**

The small population test was used (Konijn et al. 1969). 1 μl drops of amoebae
(2 x 10^4 cells) were placed on the surface of 0.5 % agarose in KK2 buffer at a
distance of 0.5–1.5 mm from an equal volume of cyclic AMP of the stated con-
centration. Plates were incubated at 22 °C for 3 h. The rate of chemotaxis was
then measured in mm h⁻¹ using an ocular micrometer.

Protein synthesis and protein glycosylation
5 μCi L(4,5-³H)leucine (40–60 Ci·mmole⁻¹) and 2 μCi D(U-¹⁴C)mannose
(200–300 mCi·mole⁻¹) were added to 10 ml of developing amoebae at t_2.
Tunicamycin in DMSO, DMSO alone or KK2 was added at t_3 then duplicate
0.5 ml samples were removed at t_4 and thereafter as stated. Nonidet P40 was
added to each sample to a final concentration of 0.2 %, samples were mixed
vigorously for 5 sec and then an equal volume of 10 % TCA containing 1 mg ml⁻¹
each of leucine and mannose was added. TCA precipitates were collected onto
Whatman GF/C filters and washed in 5 % TCA containing 1 mg ml⁻¹ each of
leucine and mannose. The filters were dried and counted in PPT scintillant
(15g PPO, 18.8 mg dimethyl POPOP per 2.5 litre toluene). The relative rates of
incorporation of [¹⁴C]mannose and [³H]leucine into TCA precipitates were
determined by double isotope analysis (Neame & Homewood, 1974) and were
used respectively as a measure of protein glycosylation and protein synthesis.

Measurements of aggregation functions
Total cell adhesion was measured by resuspending amoebae at a concentra-
tion of 10^6 cells per ml in KK2 buffer. Cell clumps were disaggregated by three
passages through a 25G x 5/8' needle. Total cell number was determined by
haemocytometer counting. The cell suspension was then shaken at 250 r.p.m.
for 30 min on a New Brunswick gyrotary shaker (model G2, 3/4' circular
orbit). The number of single cells remaining after this period was then
determined. Total cell adhesion was then calculated by subtracting the number
of single cells left after 30 min from the total number of cells and expressing
this figure as a percent of total cells. Contact sites A were measured by
performing the above assay in KK2 lacking divalent cations and in the presence
of 10 mM-EDTA (Rosen et al. 1973). A subtraction of the adhesion due to
contact sites A from total cell adhesion provided an estimate of adhesion due
to contact sites B (Marin et al. 1980). This estimate is only valid at low contact
sites A activity. Cyclic AMP phosphodiesterase was assayed as described
(Henderson, 1975). The cell-associated enzyme activity was taken as a measure
of the enzyme on the cell surface (Malchow et al. 1972) and was expressed as
milli International units (µmoles cyclic AMP hydrolysed per minute per mg
protein at 37 °C). The activity of the extracellular enzyme was expressed as
µmoles cyclic AMP hydrolysed per minute per ml of extracellular fluid at 37 °C.
Cell surface cyclic AMP receptors were assayed as described (Green & Newell,
1975).
Protein analysis and the preparation of cyclic AMP phosphodiesterase

Suspensions of developing amoebae were perfused with a solution of cyclic AMP which was delivered by an LKB microperspex pump (model 2132) at the rate of 50 µl per minute such that the concentration of cyclic AMP following the first drop was 0.2 µM. This treatment maximizes the secretion of cyclic AMP phosphodiesterase and represses the production of the protein inhibitor (Gerisch, 1979). After fifteen hours of development the suspension was cleared of cells by centrifugation at 800 g for 10 min at 22°C. The cell pellet was resuspended in 50 mM-tris-HCl, pH 7.5 (T7.5 Buffer) and the cells lysed by the addition of NP 40 to a final concentration of 0.2% (v/v). This lysate served as the source of cellular proteins. The extracellular fluid was cleared of debris by centrifugation at 10000 g for 10 min at 4°C. Protein was precipitated by the addition of ammonium sulphate to 90% saturation. The precipitate was collected, resuspended in T7.5 buffer and dialysed against 200 volumes of the same buffer. This solution provided the source for the analysis of extracellular proteins and, after passage through a column of concanavalin A Sepharose as previously described (Kessin et al. 1979), provided a partially purified extract of cyclic AMP phosphodiesterase, 30 i.u. of cyclic AMP phosphodiesterase were obtained from 100 ml of an amoebal suspension.

Protein determinations were carried out by the method of Lowry (Lowry et al. 1951) and the secreted polypeptides were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli, 1970) followed by silver staining of the separated proteins (Oakley et al. 1980).

RESULTS

The effect of tunicamycin on protein synthesis and protein glycosylation

We studied the effect of tunicamycin (TM) on the incorporation of [14C]mannose and [3H]leucine into TCA-precipitable material in order to monitor its effects on both protein synthesis and glycosylation. Mannose was chosen because it is the major sugar constituent of N-linked oligosaccharides, the synthesis of which is inhibited by TM. [14C]mannose and [3H]leucine were added to cell suspensions 2 h after the onset of development (t2). TM was then added at t3 to final concentrations ranging from 1 µg ml⁻¹ to 40 µg ml⁻¹. The amounts of radioisotopes incorporated were then determined. The results (Table 1) show that TM up to a concentration of 40 µg ml⁻¹ had no significant effect on the incorporation of [3H]leucine into TCA-precipitable material. However, increasing concentrations of the antibiotic markedly inhibited the incorporation of [14C]mannose. A concentration of 5 µg ml⁻¹ inhibited the incorporation of [14C]mannose by 50%, near maximal inhibition being obtained at concentrations of 10 µg ml⁻¹ or greater. We take these data to show that TM inhibits protein
Table 1. The effect of tunicamycin concentration on the rate of incorporation of \( ^{3}\text{H}\)leucine and \( ^{14}\text{C}\)mannose into TCA-precipitable material by amoebae from \( t_4 \) to \( t_9 \) of development

<table>
<thead>
<tr>
<th>Tunicamycin concentration (( \mu \text{g ml}^{-1} ))</th>
<th>Rate of incorporation of ( ^{3}\text{H})leucine (% control)</th>
<th>Rate of incorporation of ( ^{14}\text{C})mannose (% control)</th>
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<tr>
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<td>13</td>
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</tbody>
</table>

The conditions of labelling and analysis were as outlined in the Materials and Methods. The control rates for \( ^{3}\text{H}\)leucine and \( ^{14}\text{C}\)mannose incorporation were 135 d.p.m./\( 10^7 \) cells/h and 94 d.p.m./\( 10^7 \) cells/h, respectively.

glycosylation in *Dictyostelium* but has little or no effect on total protein synthesis. Thus, it appears that TM affects *D. discoideum* in exactly the same way that it effects other eukaryotic cells. The effect of TM on the time course of protein synthesis and glycosylation is shown in Fig. 1. There was no appreciable effect of TM on the incorporation of \( ^{3}\text{H}\)leucine (Fig. 1A), but, under the conditions of the assay, TM reduced the rate of \( ^{14}\text{C}\)mannose incorporation to 4% of the control rate. This low level incorporation of \( ^{14}\text{C}\)mannose into TCA-precipitable material in the presence of TM may reflect the general metabolism of mannose or the incorporation of the sugar into oligosaccharide chains that are attached to serine or threonine residues in proteins via O-glycosidic bonds. Such incorporation would be expected to be TM insensitive. Treatment of TCA precipitates with 25% 2-methoxyethanol (v/v) to solubilize large molecular weight carbohydrate did not reduce this low level, TM insensitive \( ^{14}\text{C}\)mannose incorporation (data not shown).

*The effects of TM on development*

When TM (10–40 \( \mu \text{g ml}^{-1} \)) was added to suspensions of developing amoebae between \( t_6 \) and \( t_4 \), and the cells then plated on agarose containing an equivalent concentration of the antibiotic, aggregation was severely impaired and all subsequent development was blocked. Amoebae failed to aggregate fully, instead, small aggregates began to form at about the same time as control amoebae aggregated (Fig. 2). The formation of these small aggregates was very rapid, proceeding from initiation to completion in 30 to 40 min (Fig. 3). These small aggregates often resembled an interconnected array of flat areas of amoebae (e.g. Fig. 2B and Fig. 4A) but further development did not occur. However, if
Tunicamycin and the development of Dictyostelium

Fig. 1. The effect of tunicamycin on the incorporation of $[^3H]$leucine and $[^14C]$mannose by developing amoebae. Labelling conditions were as described in the Materials and Methods section. (A) $[^3H]$leucine incorporation; (B) $[^14C]$mannose incorporation into TCA precipitable material. 'L' denotes the time of addition of the labelled compound and 'T' denotes the time of addition of tunicamycin to a final concentration of 10 $\mu$g ml$^{-1}$. (●—●) control amoebae; (■—■) tunicamycin-treated.

amoebae were exposed in suspension to TM from $t_0$ to $t_{10}$ then plated as 2 $\mu$l drops on agarose lacking the antibiotic, normal-looking fruiting bodies were observed 48 h later, showing that TM does not cause an irreversible block in development. TM also affected aggregation and subsequent development at concentrations below that required for complete inhibition of protein glycosylation (i.e. 10 $\mu$g ml$^{-1}$; Table 1). The small, abnormal aggregates were observed in the presence of TM at concentrations ranging from 40 ng ml$^{-1}$ to 40 $\mu$g ml$^{-1}$. Their formation began at the same time as aggregation of control amoebae and was always complete within 60 min, although cell movements were still observed after this time and the small aggregates very often fused together into an
interconnected array. This process was usually complete by ₉₈ and was most marked at TM concentrations below ₅μg ml⁻¹. No further development was observed within the flat arrays at TM concentrations above 1·25 μg ml⁻¹ but at lower antibiotic concentrations mounds of cells were observed at ₉₂₂ which were larger and more numerous at the lowest TM concentrations studied (Figs 4A, B and C). These mounds had developed between ₉₈ and ₉₂₂ by apparently drawing in amoebae from the surrounding flat arrays but they did not develop further. At the lowest TM concentrations (40 ng ml⁻¹ to 0·16 μg ml⁻¹) nearly all the amoebae present initially in the flat arrays had collected into mounds by ₉₂₂ and ‘finger-like’ projections could be seen protruding from these mounds (Fig. 4D). These structures failed to develop further. Thus it appears that TM blocked development at all the concentrations studied but that the precise point of the block depended on the concentration of TM.

The effect of TM on the development of aggregation functions

If TM (final concentration of 10 μg ml⁻¹) was added to amoebae developing in suspension they subsequently failed to acquire cell-associated cyclic AMP phosphodisterase and contact sites A activity (Fig 5A and 5B, respectively) but they did acquire near normal levels of cell surface cyclic AMP receptors (Fig. 5C) Amoebae developing on agarose containing TM also failed to accumulate
Fig. 3. Time course of appearance of the small aggregates in the presence of tunicamycin (10 μg ml⁻¹). Amoebae were allowed to develop on agar in the presence of 10 μg ml⁻¹ tunicamycin. The horizontal bar represents a distance of 40 μm. (A) Single cells observed at t₅₋₇₅, (B) aggregation beginning to take place at t₅₋₉, (C) definite aggregates observed at t₆, (D) typical final appearance of small aggregates viewed at t₆₋₅.
Fig. 4. The effect of different concentrations of tunicamycin on the development of amoebae on an agar surface. Amoebae were allowed to develop on agar containing different concentrations of tunicamycin and photographs were taken after 22 h development. Arrows indicate the mounds that occur within the flat arrays of cells. In A, B and C the magnification used was ×6 and the horizontal bar represents 2 mm; in D the magnification used was ×50 and the bar represents 40 μm. A. 5 μg ml⁻¹; B. 0.6 μg ml⁻¹; C. 0.3 μg ml⁻¹; D. 40 ng ml⁻¹.
Tunicamycin and the development of Dictyostelium

Cell-associated cyclic AMP phosphodiesterase and contact sites A (data not shown). Addition of TM to amoebae during the normal developmental increase in cyclic AMP phosphodiesterase and contact sites A activity prevented any further accumulation of these two activities (data not shown), indicating that the antibiotic has an almost immediate inhibitory effect on their expression.

We consistently observed a slight decrease in total cell adhesion during the first three hours of development which then increased as contact sites A (csA) activity increased (Fig. 6). Since the level of csA is very low during the first three hours, this decrease must be due to a slight reduction in contact site B (csB) mediated adhesion. Our estimate of csB activity is only valid at low csA activity therefore we are unable to plot csB activity at later stages of development, however, csB activity has been reported to remain fairly constant throughout development.

Fig. 5. The effect of tunicamycin on the acquisition of aggregative functions. The arrows mark the time of addition of tunicamycin to a final concentration of 10 μg ml⁻¹. All enzyme activities were assayed as outlined in the Materials and Methods section. (A) Cell-surface cyclic AMP phosphodiesterase, (B) contact sites A, (C) cell-surface cyclic AMP receptors. (●●●) control amoebae; (■■■) tunicamycin-treated.
Fig. 6. The effect of tunicamycin on cell cohesion during development. Tunicamycin was added at the beginning of development (t₀) to a final concentration of 10 μg ml⁻¹. (A) Control amoebae; (B) tunicamycin-treated. (○○○) Total cohesion, (□□□) EDTA-sensitive cohesion (contact sites B), (△△△) EDTA-resistant cohesion (contact sites A).

Fig. 7. The effect of tunicamycin and exogenous cyclic AMP phosphodiesterase (PDE) on the chemotaxis of amoebae toward cyclic AMP. Chemotaxis tests were performed as outlined in Materials and Methods section. The bar represents a distance of 2 mm. The centre of the original amoebal droplet is marked by the asterisk and the boundary of the original droplet is marked by the broken line. Tunicamycin was added at a concentration of 10 μg ml⁻¹ where appropriate. Arrows indicate the front of the responding amoebae, the cyclic AMP source being on the right of the picture in each case. (A) Chemotaxis of control amoebae toward 0.05 mM-cyclic AMP; (B) chemotaxis of control amoebae toward 5 mM-cyclic AMP in the presence of 3.6 milli i.u. of exogenous PDE; (C) the response of tunicamycin-treated amoebae toward 0.25 mM-cyclic AMP; (D) chemotaxis of tunicamycin-treated amoebae toward 5 mM-cyclic AMP in the presence of 3.6 milli i.u. of exogenous PDE.
(Beug et al. 1973). Amoebae incubated in the presence of 10 μg ml⁻¹ TM from t₀ lost all cohesion by t₃ (Fig. 6B), the rate of loss being more rapid in the presence of TM than the early loss of adhesion of control amoebae (compare Fig. 6A and 6B). Thus *de novo* protein glycosylation would appear to be necessary
not only for the developmental appearance of csA but also for the maintenance of csB activity throughout development.

The effect of TM on amoebal chemotaxis toward cyclic AMP

Figure 7 shows the effect of TM on the ability of amoebae to chemotact toward cyclic AMP as measured by the 'small population test' (Konijn et al. 1969). Amoebae were allowed to develop from t₀ in suspension in either the presence or absence of TM (10 μg ml⁻¹). At t₄ amoebae were removed from suspension and deposited on chemotaxis plates as described in the "Materials and Methods" section. Plates were examined 3 h later for signs of chemotaxis. All chemotaxis tests for TM-treated amoebae were performed on agar containing 10 μg ml⁻¹ TM. Control amoebae responded to 50 μM cyclic AMP by positive chemotaxis at a rate of 0.73 mm h⁻¹ over the 3 h period (Fig. 7A). TM-treated amoebae failed to chemotact significantly toward any cyclic AMP concentration in the range

Table 2. The rates of chemotaxis of amoebae toward different concentrations of cyclic AMP in the presence and absence of tunicamycin (10 μg ml⁻¹) and exogenous cyclic AMP phosphodiesterase (PDE)

A. Chemotaxis in the absence of cyclic AMP phosphodiesterase

<table>
<thead>
<tr>
<th>Cyclic AMP concentration (mM)</th>
<th>Chemotaxis (mm h⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>Control amoebae</td>
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<tr>
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<tr>
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<td>0.50</td>
<td>0.42</td>
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<td>0.75</td>
<td>0.34</td>
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</tbody>
</table>

B. Chemotaxis in the presence of cyclic AMP phosphodiesterase

<table>
<thead>
<tr>
<th>Cyclic AMP concentration (mM)</th>
<th>Chemotaxis (mm h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control amoebae</td>
</tr>
<tr>
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</tr>
<tr>
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<td>0.48</td>
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</table>

Chemotaxis tests were performed as outlined in the Materials and Methods section. The concentrations of cyclic AMP given are those which elicited an amoebal response under the various conditions used.

** Denotes a response of TM-treated amoebae to cyclic AMP, as discussed in the text, without measurable chemotaxis.
Fig. 8. Analysis of total secreted proteins from cells developing in the presence or absence of tunicamycin (10 μg ml⁻¹). Total secreted proteins were prepared and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis as outlined in the Materials and Methods section. Proteins were detected using the ultrasensitive silver stain method (Oakley et al. 1980). The position of migration of the different marker proteins and their relative molecular masses are given on the left-hand side of the figure and the position of the major polypeptides whose secretion is inhibited by tunicamycin (p65, p52 and p50) is indicated on the right-hand side. 5 μg of protein was applied to each track. (A) In the presence of tunicamycin (10 μg ml⁻¹), (B) in the absence of tunicamycin.
However, amoebae were consistently observed to respond to cyclic AMP in the range 0.1 mM to 0.5 mM by moving out slightly from the original confines of the droplet toward the cyclic AMP source (Fig. 7C).

The effect of exogenous cyclic AMP phosphodiesterase (PDE) on chemotaxis was also studied. Approximately 4 milli i.u. of partially purified extracellular PDE was added to the amoebal droplet used in the chemotactic tests. In the presence of PDE, the threshold cyclic AMP concentration below which control amoebae would not chemotact was raised approximately 100-fold (Fig. 7B). Addition of the enzyme to TM-treated cells resulted in amoebal chemotaxis toward cyclic AMP concentrations ranging from 0.5 mM to 50 mM (Fig. 7D). The chemotactic rates of control and TM-treated amoebae toward a range of cyclic AMP concentrations under these different conditions are summarized in Table 2. Comparison of Figs 7C and 7D also illustrates that in the presence of PDE TM-treated cells that are left within the confines of the original droplet formed into very compact, small aggregates instead of the usual rather amorphous field of small aggregates. This same difference in morphology was observed when TM-treated cells were placed on agar in the absence of a droplet of cyclic AMP (data not shown).

**Polypeptides secreted by TM-treated amoebae**

Amoebae were allowed to develop in suspension for 15 h either in the presence or absence of tunicamycin. Developing cells were perfused with cyclic AMP throughout the 15 h period in order to maximize the production of extracellular cyclic AMP phosphodiesterase (see Materials and Methods). At t15 cellular and secreted proteins were processed for analysis. TM-treated cells contained 10% more protein than controls but secreted 50% less protein than controls (data not shown). We were unable to detect any extracellular cyclic AMP phosphodiesterase activity in TM-treated cells, even after the removal of any unhydrolysed cyclic AMP from the extracellular fluid. Analysis of secreted proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis showed that TM-treated cells failed to secrete three major polypeptides under the conditions used (Fig. 8). These polypeptides had apparent relative molecular masses of 65 000 (p65), 52 000 (p52) and 50 000 (p50). No polypeptides were observed in the secreted extracts of TM-treated amoebae which were not also present in the secreted extracts of controls. There is evidence to show that p52 and p50 are closely related glycoprotein subunits of the extracellular cyclic AMP phosphodiesterase (C. J. McDonald and J. Sampson, manuscript in preparation).

**DISCUSSION**

In this paper we report that during the aggregation phase of development in *Dictyostelium discoideum* tunicamycin (TM) at concentrations above 10 μg ml⁻¹ inhibited protein glycosylation but had little or no effect on protein synthesis.
Tunicamycin and the development of Dictyostelium

This inhibition of protein glycosylation was accompanied by a reversible block in development. These data substantiate a preliminary report that TM blocked the development of *D. discoideum* (Toda *et al.* 1980). Two recent studies have shown that TM inhibited protein glycosylation in *D. discoideum* although as concentrations of only 3 μg ml⁻¹ (Lam & Siu, 1982) and 5 μg ml⁻¹ (Yamada *et al.* 1982) were used, complete inhibition was not achieved. In all other eukaryotes studied so far, TM specifically inhibited the first step of the N-asparaginyl-linked pathway of protein glycosylation (Lennarz, 1980). It is therefore reasonable to assume that TM inhibition of protein glycosylation in *D. discoideum* occurs via a similar mechanism.

The effect of TM on aggregation was very much concentration dependent. At 10 μg ml⁻¹, which caused maximal inhibition of protein glycosylation, aggregation was severely impaired and only very small aggregates developed; all further development was blocked. At TM concentrations below 10 μg ml⁻¹ protein glycosylation was incompletely inhibited and the small aggregates of cells were observed to collect together into larger interconnected arrays of cells. At TM concentrations below 1·25 μg ml⁻¹ mounds of cells developed within these interconnected arrays and at the lowest antibiotic concentrations tested (40 ng ml⁻¹) multiple ‘finger-like’ projections developed from these mounds by t₂₂, but development did not progress further. The morphology of aggregation of amoebae from the flat arrays into mounds and ‘finger-like’ structures at sub-inhibitory concentrations of TM may be grossly aberrant, but the phenomenon could represent a slower version of normal aggregation caused by the slower accumulation of glycoproteins necessary for the process. Defects in the process would arise due to glycoproteins not reaching the required concentration for the formation of normal structures and normal levels of enzyme activity. We suggest that the small aggregates which form regardless of the concentration of TM may represent a transitional stage in aggregation which is blocked from further development by the absence of specific glycoproteins which are crucial for normal development.

TM prevented the acquisition of two glycoproteins implicated in normal development:— cyclic AMP phosphodiesterase and contact sites A. Indeed, the small aggregates formed in the presence of TM were reminiscent of those formed by aggregation-deficient mutants which lack cyclic AMP phosphodiesterase (Riedel *et al.* 1973). Cyclic AMP phosphodiesterase activity has been shown to be required for normal aggregation: an aggregateless mutant deficient in the enzyme, HPX235, can aggregate when supplied with an exogenous source of cyclic AMP phosphodiesterase (Darmon *et al.* 1978), and aggregation can be blocked by a specific neutralizing anti-cyclic AMP phosphodiesterase antibody (Goidl *et al.* 1972). Amoebae acquired cyclic AMP receptors normally in the presence of TM and when PDE was added to them they regained the ability to chemotact toward cyclic AMP, formed more compact aggregates but did not develop further. We therefore suggest that the primary block in aggregation
induced by TM treatment is to prevent the synthesis of active PDE and that the subsequent block in development is perhaps caused by either a defect in regulation of the enzyme's activity or by the lack of cell cohesion due to the inhibition of the appearance of contact sites A. The formation of small aggregates which we observed on agarose containing TM may be explained by the diffusion of secreted cyclic AMP into the agarose thus preventing the immediate saturation of cyclic AMP receptors and allowing amoebae to respond to a limited number of chemotactic signals. Similar small aggregates were not observed when amoebae were spread on nitrocellulose filters (Lam & Siu, 1982) although it is interesting that in that study amoebae were observed to 'orientate' pseudopodia towards cyclic AMP in chemotactic tests.

The normal acquisition of cyclic AMP receptor activity in the presence of TM implies that de novo protein glycosylation is not necessary for the proper expression of its function. This does not rule out a regulatory role for preexisting glycoproteins such as contact sites B (Marin et al. 1980) but as cell cohesion rapidly decreased during early development in the presence of TM, if such a role existed, one might expect at least a slower accumulation of receptor activity and a reduced ability to chemotact toward cyclic AMP in the presence of exogenous PDE. The results also suggest that the cyclic AMP receptor is not a glycoprotein although it has to be borne in mind that certain glycoproteins still retain biological activity in their non-glycosylated states (Speake et al. 1981; Onishi et al. 1979). The reversibility of the inhibition of development by TM may be useful in studies of the reaggregation of cells rendered non-cohesive by the TM inhibition of expression of cohesion-mediating molecules both in Dictyostelium and in other cell types.

We would like to thank the SERC for financial support in the form of a project grant and a studentship to C.J.M. We also thank Mrs Jane Drew for her excellent technical assistance.

REFERENCES


(Accepted 18 August 1983)