Inhibition of the Development of the Cellular Slime Mould *Dictyostelium discoideum* by ω -Aminocarboxylic Acids

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SUMMARY

Four ω -aminocarboxylic acids – ϵ -aminocaproic acid (EACA), *trans*-4-aminomethylcyclohexane-1-carboxylic acid (t-AMCHA), *p*-aminomethylbenzoic acid (PAMBA) and ω -aminocaprylic acid (OACA) – prevented fruiting body formation of the cellular slime mould *Dictyostelium discoideum*. At concentrations of 40 mM, 75 mM, 10 mM and 5 mM, respectively, they allowed aggregation but prevented all further development at 24 °C. At lower concentrations, EACA allowed fruiting body formation but with a reduced number of spores per fruiting body. Only t-AMCHA had a significant inhibitory effect on the growth of myxamoebae. EACA affected development only if it was present between 8 and 16 h after the cells were deposited on the filters. Its effect was enhanced by high salt concentrations and by higher temperature, and was also dependent on the manner in which the cells were grown. Only strains capable of axenic growth displayed this sensitivity to EACA, although strains carrying only one of the genetic markers for axenic growth (*axeA*) were partially sensitive.

INTRODUCTION

When myxamoebae of the cellular slime mould *Dictyostelium discoideum* are starved, they enter the developmental phase of their life-cycle and the free-living myxamoebae aggregate to form a multicellular pseudoplasmodium or grex. The aggregated cells undergo a series of morphological changes which culminate in the formation of a fruiting body consisting of two cell types, stalk cells and spores (Garrod & Ashworth, 1973; Loomis, 1975). In the analysis of events underlying this developmental programme, inhibitors may be useful in revealing important processes: in this paper we report on a group of agents that apparently block development at an intermediate stage.

During an investigation of the effects of a number of inhibitors of proteolytic activity on the development of *D. discoideum*, it was noted that the antifibrinolytic agent ϵ -aminocaproic acid (Ablondi *et al.*, 1959; Alkjaersig, Fletcher & Sherry, 1959) did not allow development to proceed after aggregation had taken place, although it had no effect on the protein loss which accompanied development (North & Campbell, 1976). The data presented here show that the effect is not only of interest because it is stage specific, but also because it is related to the ability of strains of *D. discoideum* to grow axenically.

METHODS

Chemicals. *Trans*-4-aminomethylcyclohexane-1-carboxylic acid (t-AMCHA, tranexamic acid) was a gift from Kabi Pharmaceuticals, London W5. *p*-Aminomethylbenzoic acid

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(PAMBA) was a gift from May & Baker, Dagenham, Essex, and also from VEB Arzneimitelwerk, Dresden, East Germany. β -Alanine, γ -aminobutyric acid (GABA), δ -aminovaleric acid hydrochloride (DAVA), ϵ -aminocaproic acid (EACA), ω -aminocaprylic acid (OACA), L-lysine hydrochloride, DL-norleucine, diaminopimelic acid (DAPA), cycloheximide and streptomycin sulphate were supplied by Sigma. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was supplied by Koch-Light. All other chemicals were of the highest purity obtainable from Fisons Scientific Apparatus, Loughborough, Leicestershire.

Strains. Unless otherwise indicated, *Dictyostelium discoideum* strain AX2 (ATCC24397) was used; details of all other strains are provided in the paper.

Growth of myxamoebae. Myxamoebae were grown at 22 °C in shaken cultures in axenic medium lacking added carbohydrate, as described by Watts & Ashworth (1970). The effect of various agents on growth was tested by growing myxamoebae in 10 ml filter-sterilized axenic medium containing the particular compound in 50 ml Erlenmeyer flasks. The cultures were inoculated with myxamoebae from an exponentially-growing culture at a density of approximately 3×10^4 cells ml⁻¹, and the change in cell density was followed over a suitable period by counting the cells with a haemocytometer.

Myxamoebae were also grown in shaken cultures in association with *Klebsiella aerogenes*. Bacteria were grown on SM agar (Sussman, 1966) and harvested into 0.017 M-sodium phosphate buffer pH 6.0, to give a density of approximately 5×10^9 bacteria ml⁻¹. The suspension was inoculated with myxamoebae and incubated at 22 °C, as with axenically-grown cells. This procedure was adapted from the method of Gerisch (1960) described by Every & Ashworth (1975) for growth on *Escherichia coli* B/r. Growth tests were carried out using bacteria resuspended in sodium phosphate buffer containing the compound to be tested.

Differentiation of myxamoebae. Myxamoebae were grown in 660 ml axenic medium or in 700 ml suspensions of *K. aerogenes* in 2 l Erlenmeyer flasks as described above. They were grown axenically unless otherwise indicated. The cells were harvested in the exponential phase of growth, washed with ice-cold distilled water, deposited on Millipore filters and allowed to differentiate as described by Hames & Ashworth (1974). When additions were made to the pad diluting fluid (PDF; Garrod & Ashworth, 1972), sufficient material was added to give a concentration 1.31 times greater than the final concentration required: this allowed for the dilution of the PDF by the 0.5 ml fluid in which the cells were deposited.

Harvesting and counting of spores. Spores were harvested from mature fruiting bodies by touching the spore masses with a fine capillary drawn from a Pasteur pipette: the spores collected in the capillary. Spores were picked from 100 fruiting bodies taken at random, resuspended in 1 ml water (or 10 mM-potassium phosphate buffer pH 6.5, if the heat stability of the spores was to be tested; North & Campbell, 1976), and counted with a haemocytometer. Spore populations from fruiting bodies formed after certain treatments contained a small (less than 10%) proportion of non-refractile cells: these were not scored as spores.

Testing strains for sensitivity to EACA. Suspensions of spores of different strains of *D. discoideum* were plated with *K. aerogenes* on SM agar containing 50 mM-EACA. In general, the bacterial suspension (0.2 ml) was spread on the plate, and after 15 min a small volume (10 to 40 μ l) of spore suspension was placed in the centre. Plates were incubated at 24 °C and observations made after 5 to 7 days: the sensitivity to EACA could be determined from the extent of fruiting body formation. Control plates lacking EACA were always set up in parallel.

Selection of haploid segregants from diploid strains. Haploid segregants were isolated from the diploid strains DP49 and DP59 (Williams, Kessin & Newell, 1974a) by plating spores

with *K. aerogenes* on SM agar containing cycloheximide ($400\ \mu\text{g ml}^{-1}$) or methanol (2 %, v/v) at a density of about 10^4 spores per plate, as described by Gingold & Ashworth (1974). The size of the spores from resistant clones was examined microscopically to distinguish the haploid segregants from resistant diploids (which arise by mitotic recombination; Gingold & Ashworth, 1974).

Characterization of strains. Strains were characterized on the basis of their ability to grow with *K. aerogenes* on SM agar containing cycloheximide ($400\ \mu\text{g ml}^{-1}$) or methanol (2 %, v/v) at 22 °C and on SM agar plates at 27 °C, and also on the basis of pigmentation (*whi* and *bwn*; Williams *et al.*, 1974*a*). The ability of strains to grow axenically was tested by adding approximately 10^4 spores or myxamoebae to axenic medium (1 ml) containing streptomycin ($250\ \mu\text{g ml}^{-1}$; Watts & Ashworth, 1970) in 100×12 mm test-tubes. The tubes were rotated in a simple roller apparatus at 15 rev. h^{-1} with the tubes at an angle of approximately 10° to the horizontal. Growth was assessed during a period of up to 7 days incubation at 22 °C.

RESULTS

The EACA effect

When axenically-grown myxamoebae of *D. discoideum* AX2 were incubated on Millipore filters at 24 °C in the presence of the antifibrinolytic agent EACA at concentrations above 40 mM, the cells aggregated in the same time (10 to 12 h) as did cells not exposed to EACA. However, subsequent morphological changes did not occur, and the cells either remained as large flat aggregates, or formed into smaller aggregates (Fig. 1*d*). There was no formation of a tip. Clonal plating of suspensions of EACA-treated cells with *K. aerogenes* on SM agar gave 100 % viable cells, even after 48 h on Millipore filters. Development was affected by concentrations of EACA as low as 5 to 10 mM. The cells were able to form fruiting bodies but the spore masses of these were translucent rather than opaque. As the EACA concentration was increased, so the effect on development became more severe, and the final structure formed was less like the normal fruiting body.

Figure 1 shows the effect of increasing EACA concentrations on development, and the predominant types of structure observed are shown diagrammatically in Fig. 2. Structure C apparently resulted from the folding-over of the spore mass, since the stalk was seen to be hooked around the spores. In both structure C and structure D, which had no spore mass, the presumed stalk was much broader than normal. Structure E resembled a normal slug to some extent, but was notable for the absence of a well-defined tip.

The increased translucence of the spore masses with increasing EACA concentrations suggested that the number of spores was reduced, although visual observation revealed that the fruiting body size remained approximately the same. This was confirmed by harvesting spores from fruiting bodies formed in the presence of different concentrations of the inhibitor (Fig. 3). In addition to this reduction in the number of spores, it was found that, although cells were picked only from the tip of the stalk, there was a small but significant number (up to 10 % of the total) of non-refractile and apparently undifferentiated cells present at high EACA concentrations. The spore mass also became increasingly sticky.

An identical effect was observed with other, related amino acids (Table 1). Two other antifibrinolytic agents, t-AMCHA and PAMBA (Okamoto *et al.*, 1968; Donner & Housková, 1967), also blocked development of *D. discoideum* although aggregation was apparently normal. The concentrations required for a complete block after aggregation differed from that required with EACA (above 75 mM-t-AMCHA; above 10 mM-PAMBA). Only one of the homologues of EACA tested was effective in blocking development in a similar manner.

Fig. 1. Effect of EACA on the development of axenically-grown myxamoebae of *Dictyostelium discoideum* strain AX2 on Millipore filters. The cells were incubated at 24 °C for 48 h in the presence of EACA at the following concentrations : (a) 0 mM, (b) 17.5 mM, (c) 35 mM, (d) 50 mM. Bar marker represents 1 mm.

Fig. 2. Diagrammatic representations of the final structures formed by axenically-grown myxamoebae incubated at 24 °C on Millipore filters with different concentrations of EACA.

Fig. 3. Effect of EACA concentration on the number of spores per fruiting body. Spores were harvested from fruiting bodies formed by axenically-grown myxamoebae deposited on Millipore filters and incubated at 24 °C for 28 h (○), 40 h (●) or 44 h (□). The values are averages from 2×100 fruiting bodies (●, □) or 5×100 fruiting bodies (○).

At low concentrations (5 to 10 mM), ω -aminocaproic acid (OACA) had the same effect as 40 mM-EACA ; but, at higher concentrations it also inhibited aggregation. δ -Aminovaleric acid was also an inhibitor of development but inhibited aggregation, which suggested that it may not have had the same effect as EACA. All the other ω -aminocarboxylic acids tested allowed fruiting body formation when present at 50 mM, although the morphology of the fruiting bodies and the exact time of their completion was sometimes found to differ from that of the controls. This was particularly so with lysine, which at high concentrations (100 mM) caused very sparse development. None of the ω -aminocarboxylic acids caused the specific block after aggregation observed with EACA.

At concentrations above 5 mM, norleucine (α -aminocaproic acid) completely inhibited development, including aggregation ; and at lower concentrations (1 to 5 mM), development was delayed and fruiting body formation became sparser with increasing concentration. No effect analogous to that of EACA was observed.

Neither lysine nor diaminopimelic acid was able to overcome the block caused by 50 mM-EACA when they too were added to PDF at a concentration of 50 mM.

Table 1. *Effect of ω -aminocarboxylic acids on the development of myxamoebae on Millipore filters at 24 °C*

Amino acids were added to PDF: for DAVA, the pH was adjusted to 6.5 with NaOH. Axenically-grown myxamoebae were deposited and incubated at 24 °C. Observations were made over a period of 48 h.

Amino acid	Structure	Effect on aggregation*		Effect on fruiting†	
		10 mM	50 mM	10 mM	50 mM
Glycine	$\text{NH}_2\text{CH}_2\text{COOH}$	+	+	+	+
β -Alanine	$\text{NH}_2(\text{CH}_2)_2\text{COOH}$	+	+	+	+
GABA	$\text{NH}_2(\text{CH}_2)_3\text{COOH}$	+	+	+	+
DAVA	$\text{NH}_2(\text{CH}_2)_4\text{COOH}$	+	—	+	—
EACA	$\text{NH}_2(\text{CH}_2)_5\text{COOH}$	+	+	+	—
OACA	$\text{NH}_2(\text{CH}_2)_7\text{COOH}$	+	—	—	—
Norleucine	$\text{CH}_3(\text{CH}_2)_3\text{CHNH}_2\text{COOH}$	—	—	—	—
Lysine	$\text{NH}_2(\text{CH}_2)_4\text{CHNH}_2\text{COOH}$	+	+	+	+
DAPA	$\text{COOHCHNH}_2(\text{CH}_2)_3\text{CHNH}_2\text{COOH}$	+	+	+	+
t-AMCHA	<i>trans</i> - $\text{NH}_2\text{CH}_2\text{C}_6\text{H}_{10}\text{COOH}$	+	+	+	—
PAMBA‡	$\text{NH}_2\text{CH}_2\text{C}_6\text{H}_4\text{COOH}$	+	+	—	—

* +, Aggregation took place as in controls; —, no aggregation after 48 h.

† +, Fruiting body formation took place, although not necessarily in the same manner as controls; —, no fruiting bodies formed.

‡ No NaCl present.

Effect of inhibitors on growth

The lack of effect of EACA, t-AMCHA and PAMBA on the early stages of development suggested that they probably do not act as general inhibitors of metabolism. At concentrations that blocked development, neither EACA, PAMBA nor OACA had a significant effect on growth of myxamoebae in axenic medium or with suspensions of *K. aerogenes*. t-AMCHA was inhibitory, particularly when the myxamoebae were grown with bacteria, but this may have been a reflexion of the high concentration used (75 mM).

This lack of effect on growth meant that cells could be grown with *K. aerogenes*, which was not sensitive, on SM agar to which an ω -aminocarboxylic acid had been added, and the effect of the inhibitor on development could be observed.

Time of effect of EACA

To test whether the effect of EACA on post-aggregation events required the exposure of the cells to PDF containing the inhibitor over the whole period of incubation, cells were incubated in the absence of EACA for different times and were then transferred to PDF containing 50 mM-EACA. Even when cells were transferred as late as 8 h after deposition, EACA was still able to exert its full effect (Table 2). After 10 h incubation, EACA had less effect, and failed to prevent fruiting body formation in cells exposed to it after 16 h.

When cells were transferred from EACA, the inhibitor affected development only if they were transferred at least 8 h after deposition. Even when post-aggregation development had been blocked, the removal of EACA still allowed some escape from inhibition. However, the longer the exposure to EACA the less able were the cells to recover and develop normally.

Factors affecting the sensitivity to EACA

Ionic strength and pH. At 50 mM, EACA was as effective in blocking post-aggregation development when it was dissolved in water as it was when dissolved in PDF, but the effect

Table 2. *Effect of exposure to EACA during a limited period of the development phase*

Axenically-grown myxamoebae were deposited on Millipore filters supported by pads containing PDF with and without 50 mM-EACA. At different times during incubation at 24 °C, the cells were transferred: cells incubated without EACA were transferred to EACA, and EACA-treated cells were transferred to PDF without EACA. Two transfer methods were used: (i) filters and cells were transferred directly to fresh pads and PDF; (ii) cells were washed from filters, disaggregated by trituration, collected by centrifugation, resuspended in water, and deposited on fresh filters, pads and PDF. The final structures formed were as shown in Fig. 2.

		Structure observed after 36 h total incubation on filters	
Time of transfer		Direct transfer	Disaggregation
To EACA:	6 h	G	G
	8 h	G	G
	10 h	C, D, E	D, E, F
	12 h	B	B, C
	14 h	B	A, B
	16 h	A	A
	18 h	A	A
	20 h	A	Recapitulation not possible
	24 h	A	Recapitulation not possible
	Not transferred	A	—
From EACA:	6 h	A	A
	8 h	B	A
	10 h	B	B
	12 h	C, D, E	B
	14 h	C, D, E	B
	16 h	C, D, E	B
	18 h	C, D, E	Miniature fruiting bodies
	20 h	C, D, E	Miniature slugs and aggregates
	24 h	F	G
	Not transferred	G	—

was enhanced by high ionic strength. Salts were added to PDF containing EACA, and with final salt concentrations above 20 mM (in addition to the 20 mM-KCl and 5 mM-MgCl₂ present in PDF), the concentration of EACA required to block development after aggregation decreased. For example, the presence of 40 mM-NaCl reduced the inhibitory concentration of EACA at 24 °C from 40 mM to less than 20 mM. This salt concentration did not affect the development of cells incubated without EACA. The enhancement was not apparently due to one specific ion or group of ions since it was observed with salts with different combinations of monovalent and divalent ions, e.g. NaCl, Na₂SO₄, NaNO₃, MgCl₂, KCl, NH₄Cl.

The effect of the ionic strength was particularly important in the case of PAMBA. The product supplied by VEB Arzneimittelwerk was in an isotonic solution, and so PDF containing PAMBA from this source also contained NaCl. For this reason, it was apparently more effective than the solid PAMBA supplied by May & Baker.

At 50 mM, EACA always produced a block after aggregation in the pH range 5.8 to 8.5.

Temperature. As the incubation temperature was lowered, so the concentration of EACA required to prevent post-aggregation development increased (Fig. 4). Cells incubated at 19 °C in the presence of concentrations of EACA which inhibited at 24 °C, escaped inhibition and formed the structures observed with lower EACA concentrations at 24 °C. For example, with 50 mM-EACA translucent fruiting bodies (structure B, Fig. 2) were formed. A temperature dependence was also observed for the inhibitory effects of t-AMCHA, PAMBA and OACA. Although at 24 °C, 50 mM-OACA completely inhibited aggregation,

Fig. 4. Effect of temperature on the concentration of EACA required to prevent further development of axenically-grown cells after aggregation. The concentrations are the lowest at which only structure G (Fig. 2) was formed.

this inhibition was removed by lowering the temperature to 19 °C, and at the latter temperature 50 mM-OACA had the same effect as 50 mM-EACA at 24 °C.

The temperature routinely used in these experiments (24 °C) was chosen because it allowed EACA to block development at a lower concentration than at 22 °C (the growth temperature), but also allowed temperature-sensitive growth mutants which are unable to grow at higher temperatures to be studied.

Growth conditions for myxamoebae. In addition to extracellular factors obtaining during incubation of cells on Millipore filters, the sensitivity to EACA was also dependent on the manner in which the cells were grown. Cells grown in axenic medium were inhibited by 40 mM-EACA at 24 °C (Fig. 4); but, if glucose was added to the growth medium, the concentration of EACA necessary to achieve inhibition of fruiting body formation increased, provided the cells were harvested during the exponential growth phase. Cells grown in the presence of 86 mM-glucose escaped complete post-aggregation inhibition at EACA concentrations of less than 80 mM. However, if the cells were allowed to enter stationary growth phase before harvesting, the inhibitory EACA concentration was only 30 to 40 mM.

The sensitivity to EACA of cells grown in association with *K. aerogenes* in shaken culture varied from experiment to experiment, and concentrations of the inhibitor of between 40 mM and 100 mM were required to block post-aggregation development. When cells were plated in association with bacteria on SM agar containing EACA, a concentration of 50 mM always prevented development after aggregation.

The relationship between sensitivity to EACA and the ability to grow axenically

The effect of EACA was first observed with strain AX2 which was isolated for its ability to grow axenically (Watts & Ashworth, 1970). Strain NC4 (Raper, 1935), the non-axenic strain to which AX2 can be traced, was not sensitive to EACA. A difference in sensitivity between the two strains was also observed with PAMBA, t-AMCHA, and OACA. Concentrations of all four compounds (EACA, 100 mM; PAMBA, 50 mM; t-AMCHA, 80 mM; OACA, 50 mM)

Table 3. *Sensitivity of various strains to EACA*

Strain	Reference*	Genotype†	Sensitivity to EACA‡
NC4	1	+	R
VI2	2	+	R
TS12	3	+	R
NP14	4	+	R
X9	4	+	R
AX2	5	<i>axe</i>	S
AX3	6	<i>axe</i>	S
X2	4	<i>axe</i>	S
X20	4	<i>axe</i>	S
G82	7	<i>axe</i>	S§
G88	8	<i>axe</i>	S§
DP49	4	<i>axe</i> /+	R
DP59	4	<i>axe</i> /+	R

* 1, Raper (1935); 2, Erdos, Raper & Vogen (1973); 3, Katz & Sussman (1972); 4, Williams *et al.* (1974a); 5, Watts & Ashworth (1970); 6, Loomis (1971); 7, E. Gingold, growth temperature-sensitive, cycloheximide-resistant mutant from AX2; 8, Gingold (1974).

† +, Wild type, incapable of axenic growth; *axe*, able to grow axenically; *axe*/+, diploid heterozygous for axenic markers, incapable of axenic growth.

‡ R, Resistant, formed normal fruiting bodies in the presence of 50 mM-EACA at 24 °C; S, sensitive, failed to form fruiting bodies in the presence of 50 mM-EACA at 24 °C.

§ From observations of development of axenically-grown cells on Millipore filters: growth on agar plates at 24 °C was poor.

that inhibited bacterially-grown cells of AX2 allowed fruiting bodies to form from NC4 cells. It is noteworthy that, like AX2, NC4 was sensitive to norleucine, which suggests that the action of this amino acid was not related to that of EACA. δ -Aminovaleric acid (DAVA) did not block the development of NC4 cells, and in view of its effect on AX2 cells (Table 1) it is possible that DAVA may have had an effect related to that of EACA.

A second axenic strain, AX3, independently isolated (Loomis, 1971), was also sensitive to EACA, PAMBA, t-AMCHA and OACA at similar concentrations to those that inhibited AX2 cells. The inhibition of AX3 was also temperature-dependent. A number of other strains were tested for their sensitivity to EACA; all non-axenic strains were able to form fruiting bodies in the presence of 50 mM-EACA at 24 °C, but all axenic strains were inhibited (Table 3). The presence or absence of any other known marker was not a requirement for sensitivity.

The ability to grow axenically is a recessive character (Williams, Kessin & Newell, 1974b), and so diploid strains constructed between non-axenic and axenic haploid strains are incapable of axenic growth. Two such diploids were tested for their sensitivity to EACA, and both were resistant (Table 3). DP49 and DP59 were derived from the non-axenic strain TS12 and the axenic strains NP59 and NP58 respectively (Williams *et al.*, 1974a). TS12 was tested and was resistant to EACA; but strain MNA1 isolated from DP49 in this laboratory and having the identical phenotype to NP59 (methanol resistance, temperature-sensitive growth, brown pigmentation, axenic growth), was sensitive to EACA.

Proposed markers for axenic growth have been assigned to two linkage groups, *axeA* to linkage group II and *axeB* to linkage group III (Williams *et al.*, 1974a). In an attempt to determine whether one or both of these markers were required for EACA sensitivity, we isolated haploid segregants of DP49 and DP59 in which either the axenic linkage group II or both axenic linkage groups II and III were present (Table 4). The presence of these linkage groups was assigned on the basis of the inheritance of other markers. Strains with *axeA* only were selected as methanol-resistant strains which grew at 27 °C, and those with both markers

Table 4. *Haploid segregants isolated from DP49 and DP59*

Segregants from DP49 were selected initially on SM agar plus methanol (2 %, v/v) and those from DP59 on SM agar plus cycloheximide (400 µg ml⁻¹).

		Linkage group								No. tested	
Derived from		I	II				III		IV		
Diploid											
DP49 (DP59)	TS12	<i>cycA1</i>	<i>tsgD12</i>	+	<i>whi</i>	+	+	+	+		
	NP59 (NP58)	+	+	<i>acrA9(A8)</i>	+	<i>axeA1</i>	<i>axeB1</i>	<i>tsgA1</i>	<i>bwn</i>		
Haploid segregants											
Non-axenic (<i>axeA</i>)	DP49	{	+	+	<i>acrA9</i>	+	<i>axeA1</i>	+	+	3	
			+	+	<i>acrA9</i>	+	<i>axeA1</i>	+	+	<i>bwn</i>	7
	DP59	{	<i>cycA1</i>	+	<i>acrA8</i>	+	<i>axeA1</i>	+	+	+	4
			<i>cycA1</i>	+	<i>acrA8</i>	+	<i>axeA1</i>	+	+	<i>bwn</i>	5
Axenic (<i>axeA</i> , <i>axeB</i>)	DP49		+	+	<i>acrA9</i>	+	<i>axeA1</i>	<i>axeB1</i>	<i>tsgA1</i>	<i>bwn</i>	1

Gene symbols: *cycA1*, growth in the presence of cycloheximide (400 µg ml⁻¹); *tsgD12*, *tsgA1*, displays temperature-sensitive growth; *acrA8*, *acrA9*, growth in the presence of acriflavin (100 µg ml⁻¹) and methanol (2 %, v/v); *axeA1*, *axeB1*, growth in axenic medium if both markers present; *whi*, white spore; *bwn*, formation of brown pigment.

as methanol-resistant strains unable to grow at 27 °C. Attempts to isolate strains which possessed the *axeB* marker only (methanol-sensitive, white, non-leaky temperature-sensitive growth) were unsuccessful.

All of the strains tested which possessed the *axeA* but not the *axeB* marker were unable to form fruiting bodies in the presence of EACA. However, their sensitivity to EACA was intermediate between that of strains AX2 and NC4. Thus, in the presence of 50 mM-EACA, 14 out of 19 strains tested formed finger-like structures (structure D, Fig. 2). The five remaining strains were apparently as sensitive to EACA as was strain AX2, but also showed a markedly more temperature-sensitive developmental pattern such that at 24 °C in the absence of EACA fruiting body construction did not take place. We feel confident, therefore, that the sensitivity to EACA shown by these strains is due, not only to the presence of the *axeA* marker, but also to the presence of another marker not detectable in the strain (DP59) from which these five were derived.

All strains with the *axeA* marker only were unable to grow axenically. The one haploid strain isolated with both *axeA* and *axeB* markers was able to grow axenically, and was sensitive to EACA to the same degree as was strain AX2. These observations were consistent with the view that *axeA* confers sensitivity to EACA but that for complete sensitivity (as shown by strain AX2), the presence of a second marker, such as *axeB*, is also required; although more detailed genetic analysis will be necessary to show that the markers for EACA sensitivity and those for axenic growth are allelic.

In a preliminary investigation of the possibility of isolating mutants with altered sensitivity to EACA, a strain characterized as possessing the *axeA* marker only (MNA13, derived by haploidization of DP49), which formed fingers (structure D, Fig. 2) in the presence of 50 mM-EACA, was mutagenized with NTG as described by Yanagisawa, Loomis & Sussman

(1967). Mutant strains that were able to form fruiting bodies in the presence of 50 mM-EACA arose at a frequency of approximately one in 1000 amongst the survivors. Mutants were also isolated whose morphology in the presence of 50 mM-EACA was the same as that observed with AX2 but, in most cases, these strains were unable to complete development in the absence of EACA. However, we isolated two strains (MNF5, MNF20) that displayed this phenotype only in the presence of EACA. In addition to their increased sensitivity to EACA, these strains were also capable of axenic growth, unlike the parent strain (MNA13). This suggests that they arose as a result of mutation in the proposed *axeB* gene.

DISCUSSION

On the basis of the results presented in this paper, the effect of EACA and related ω -aminocarboxylic acids has been characterized as an inhibition of the development of *D. discoideum* after aggregation that is temperature dependent and related to the presence of genetic markers for axenic growth in a particular strain. A mutant of NC4 unable to develop after the aggregation stage was isolated in 1953 (Sussman & Sussman, 1953) and had a phenotype similar to that shown by strain AX2 developing in the presence of EACA. The EACA effect was specific with regard to the inhibitory compound since similar amino acids (glycine, β -alanine, γ -aminobutyric acid and lysine) could be added to PDF without producing the same effect. This ruled out the possibility that the effect was due to a simple change in the ionic conditions, as did the fact that both PAMBA and OACA inhibited at concentrations that were much lower than those needed with EACA and t-AMCHA. The time of the effect also appeared to be specific; EACA had to be present during the period 8 to 16 h after deposition on Millipore filters to exert any effect. At this time the aggregated cells would normally be starting the series of morphological changes resulting in fruiting body construction. EACA has no effect on biochemical changes occurring early in development, but severely inhibits later events (North & Campbell, 1976).

With the exception of OACA, those amino acids that blocked development after aggregation are potent inhibitors of fibrinolysis (Okamoto *et al.*, 1968). In that process they act by inhibiting the proteolytic activation of plasminogen or the action of plasmin on fibrin (Brockway & Castellino, 1971; Iwamoto, 1975). However, no other proteolytic inhibitors have been found which have the same effect on *D. discoideum* development (unpublished observations) and so the ω -aminocarboxylic acids may not necessarily act by inhibition of a proteolytic event. Reports of effects of EACA on other systems have been made, for example, the inhibition of the response of lymphocytes to phytohaemagglutinin (Hirschhorn *et al.*, 1971) and an effect on potassium metabolism in dogs (Carroll & Tice, 1966).

Whatever the mechanism for EACA inhibition of development, the difference in sensitivities between different strains must be explained. Although differences between NC4 and AX2 cells in sensitivity to certain agents have been noted previously (e.g. Weeks & Weeks, 1975), this is the first case in which a difference, apart from the ability to grow axenically, has been shown to be related to the presence of the genetic markers for axenic growth. It is anticipated that the sensitivity to EACA may provide a useful characteristic for the isolation of strains possessing axenic growth markers. In particular it may be possible to characterize strains carrying the *axeA* marker. The differences in sensitivity might also help to establish the molecular basis of the ability to grow axenically.

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