Genetic Analysis and Phenotypic Characterization of Effects on the Cytoskeleton of Coumarin-sensitivity Mutations in *Dictyostelium discoideum*

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Five coumarin-sensitivity mutations were assigned to loci on three linkage groups: *couB352* to group I; *couC356*, *couE353* and *couF354* to group II; and *couD355* to group III. Coumarin inhibited cell division, affected cell shape and induced EDTA-sensitive cell–cell adhesion in vegetative cells at lower concentrations in coumarin-sensitive mutants (1.0 to 2.5 mM) than in wild-type strains (3.0 mM). One mutant, HU609 (*couB352*), lysed in the presence of coumarin (1.6 mM). Coumarin, thiabendazole, and cambendazole induced multiple tips in pseudoplasmodia. Coumarin, unlike thiabendazole and cambendazole, did not induce metaphase arrest or haploidization of diploids. The *couA351* and *couF354* coumarin-sensitivity mutations are linked to growth temperature-sensitivity mutations, *tsgK21* and *tsgT360* respectively, which may be pleiotropic effects of the coumarin-sensitivity mutations. Exposure of vegetative cells of strains carrying either the *couA351* or the *couF354* mutation to the restrictive temperature (28 °C) induced cell rounding and cell–cell adhesion similar to that seen on treatment with coumarin. Wild-type strains, other coumarin-sensitive strains and other temperature-sensitive strains did not exhibit this phenotype at 28 °C. Furthermore, temperature-sensitivity and coumarin-sensitivity co-reverted in temperature-resistant derivatives selected from strains carrying the *couA351/tsgK21* mutation.

INTRODUCTION

Coumarin is a naturally occurring compound found in many species of plants. Coumarin and its derivatives have a broad range of effects on fungi, bacteria, algae, plants and animals (for reviews, see Wolf, 1974; Feuer, 1974; Grigg, 1978; Cohen, 1979). Of relevance to the work reported here are the effects of coumarin on processes which involve microtubules. While we know of no direct evidence that coumarin binds to tubulin or microtubule-associated proteins, it has been shown to induce metaphase arrest in cells of lily and onion (Cornman, 1947; Östergren, 1948). It also disrupts cell division and cell elongation in roots of wheat and corn, possibly by affecting the orientation of microtubules (Svensson, 1971). Other cytostatic and antifungal effects of coumarin have also been proposed to result from disruption of microtubules or microtubule organizing centres (Fasulo *et al.*, 1979; Dall'Olio & Vannini, 1979).

We have recently described a coumarin-sensitivity mutation (*couA351*) in *Dictyostelium discoideum* (Welker & Williams, 1980c). Mutations affecting sensitivity to, or metabolism of, coumarin or its derivatives are known in other species, particularly mammals, including mouse (Lush & Andrews, 1978) and man (O'Reilly & Aggeler, 1965) where coumarin derivatives are used as anticoagulants. In this report we describe the isolation and genetic characterization of five additional coumarin-sensitivity mutations in *D. discoideum*. Such mutations may affect cytoskeletal proteins, coumarin metabolism, or the permeability of the

plasma membrane to coumarin. Evidence is presented which indicates that the coumarin-sensitivity of strains which carry the *couA351* or *couF354* mutations and a linked temperature-sensitivity (*tsgK21* or *tsgT360*, respectively) may be the pleiotropic effects of single mutations (on linkage groups VII and II, respectively). We also describe the effects of coumarin on cell division, cell shape and cell-cell adhesion in mutant and wild-type vegetative cells, and on the induction of multiple tips in pseudoplasmodia.

METHODS

Growth of amoebae. Stock cultures of *Dictyostelium discoideum* were maintained in association with a cobalt-resistant strain of *Klebsiella aerogenes* at $21 \pm 1^\circ\text{C}$ on SM agar plates (Williams, 1978).

Genetic analysis. Diploids were selected on the basis of complementation of temperature-sensitivity mutations (*tsg*) and mutations that lead to the inability to grow using *Bacillus subtilis* as a food source (*bsg*) (Newell *et al.*, 1977). The restrictive temperature used for genetic analysis was $26.5 \pm 0.2^\circ\text{C}$. Complementation of the coumarin-sensitivity mutations was shown by selection of coumarin-resistant diploids on SM agar plates containing either 1.0 or 1.3 mM-coumarin. For linkage analyses, haploid segregants were obtained from heterozygous diploids that had been plated clonally on SM agar plates containing the haploidizing agent benlate ($35\text{ }\mu\text{g ml}^{-1}$) (Williams & Barrand, 1978). The genotypes of the segregant haploids were determined using previously described techniques (Newell, 1978; Welker & Williams, 1980c). The coumarin-sensitivity of segregant haploids was normally tested on SM agar plates containing 1.0 and 1.3 mM-coumarin (Welker & Williams, 1980c). Some variation in toxicity of these media was observed. Therefore plating efficiencies varied somewhat with different batches of media; those presented were obtained from representative experiments and are given as the ratios of colony-forming ability on SM agar containing coumarin relative to that on SM agar without coumarin. Haploid strains used in this work are described in Table 1; all were derived from the NC4 wild-type strain.

Chemicals. Coumarin (1,2-benzopyrone), cycloheximide and isopropyl *N*-(3-chlorophenyl)carbamate (CIPC) were obtained from Sigma. Benlate was a gift from DuPont; methanol and cobaltous chloride were analytical reagent grade from May & Baker. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was obtained from Aldrich. Thiabendazole and cambendazole were gifts from William Bliss of Merck, Sharp & Dohme, Research Labs, Ingleburn, N.S.W., Australia.

Mutagenesis. To induce coumarin-sensitivity or temperature-sensitivity mutations, amoebae of the haploid strain HU526 were treated for 30 min at $21 \pm 1^\circ\text{C}$ with either 300 or 600 $\mu\text{g MNNG ml}^{-1}$. For MNNG treatment, vegetative cells were harvested from SM agar plates, the bacteria were removed by differential centrifugation and the cells were resuspended in phosphate-buffered saline (PBS; Deering *et al.*, 1970) at 10^7 amoebae ml^{-1} . Following mutagenesis the cells were washed three times by centrifugation and then inoculated on SM agar plates to obtain colonies from the surviving cells. Survival was 12% after treatment with 300 $\mu\text{g MNNG ml}^{-1}$ and 1–3% after treatment with 600 $\mu\text{g MNNG ml}^{-1}$. Cells from these colonies were transferred with toothpicks to SM agar plates containing 1.0 mM-coumarin, or to SM agar plates which were then incubated at $26.5 \pm 0.2^\circ\text{C}$, in order to isolate coumarin-sensitive or temperature-sensitive mutants, respectively. Colonies unable to grow under these conditions were retested after cloning from the original plates which had been stored at 4°C during the initial screening period.

Selection of temperature-resistant revertants. Revertants were selected from strains bearing the *tsgK21/couA351* mutation(s) by plating between 10^4 and 10^5 amoebae or spores on SM agar plates in association with *K. aerogenes* and then allowing the cells to multiply at the permissive growth temperature (21°C) for about 2 d. When extensive growth was visible but prior to clearing of the bacterial lawn, the plates were shifted to the restrictive temperature (26.5°C). Plaques appeared on these plates over the next week. Usually one or two revertants were found per plate. Cells from these colonies were cloned once at 26.5°C and subsequently handled at 21°C .

Treatment with coumarin and mitotic inhibitors in suspension cultures. Cells were grown overnight in suspension cultures (5–15 ml in 125 ml Erlenmeyer flasks) with *Escherichia coli* B/r (about 10^{10} bacteria ml^{-1}) in PBS at 180 rev. min^{-1} on a Kühner Lab-Shaker at $21 \pm 1^\circ\text{C}$. The exponentially growing culture was then divided into control and drug-treatment cultures, diluted to between 10^5 and 5×10^5 cells ml^{-1} by the addition of fresh *E. coli* B/r suspension in PBS and returned to the shaker. Coumarin stock solutions (5 to 15 mM) were sterilized by autoclaving in PBS. Thiabendazole, cambendazole and CIPC stocks at 20 mg ml^{-1} were prepared in dimethyl sulphoxide and stored at -20°C . The drug solutions were added directly to the suspension cultures to give the final drug concentrations required. In control cultures, concentrations of dimethyl sulphoxide as high as 4.0% (v/v) had no effect on doubling time or cell-cell adhesion. Samples were removed during the course of the drug treatment to determine cell densities using a haemocytometer. Cell shape and cell-cell adhesion were observed in

Table 1. Genotypes of haploid strains of *Dictyostelium discoideum* used

Strain	Parent*	<i>acr</i>	<i>ars</i>	<i>axe</i>	<i>bsg</i>	<i>bwn</i>	<i>cob</i>	<i>cou</i>	<i>cyc</i>	<i>ebr</i>	<i>frt</i>	<i>man</i>	<i>sup</i>	<i>tsg</i>	<i>whi</i>	Ref.†
HL33	HL21	+	+	+	B500	+	+	+	+	+	+	+	+	P500	B500	1
HU215	DU336	C4	B353	BI	+	+	+	+	+	+	+	+	+	D12	AI	2
HU413	HU407	A371	+	+	A5	AI	+	A351	AI	+	B353	+	+	K21	+	3
HU526	DU740	+	+	+	A5	AI	+	+	AI	+	+	A2	+	+	+	2
HU528	DU780	+	+	+	A5	+	AI	+	AI or A5	+	+	A2	+	D12	AI	2
HU606	HU526	+	+	+	A5	AI	+	+	AI	+	+	A2	+	Q356	+	2
HU607	HU526	+	+	+	A5	AI	+	+	AI	+	+	A2	+	M357	+	2
HU608	HU526	+	+	+	A5	AI	+	C356	AI	+	+	A2	+	R358	+	2
HU609	HU526	+	+	+	A5	AI	+	B352	AI	+	+	A2	+	+	+	2
HU610	HU526	+	+	+	A5	AI	+	E353	AI	+	+	A2	+	+	+	2
HU611	HU526	+	+	+	A5	AI	+	F354	AI	+	+	A2	+	T360	+	2
HU612	HU526	+	+	+	A5	AI	+	D355	AI	+	+	A2	+	+	+	2
HU795	DU1085	AI	+	+	+	+	+	B352	AI	+	+	A2	+	D12	AI	2
HU799	HU610	AI808	+	+	A5	AI	+	E353	AI	+	+	A2	+	+	+	2
HU833	DU1088	AI	+	+	A5	AI	+	D355	+	+	+	+	+	D12	AI	2
HU836	DU1104	+	+	BI	+	AI	+	F354	AI	+	+	+	+	AI, T360	+	2
HU881	HU606	AI812	+	+	A5	AI	+	+	AI	+	+	A2	+	Q356	+	2
HU960	HU876	AI814	+	BI	+	AI	+	F354	AI	+	+	+	+	AI, T360	+	2
HU970	HU876	AI816	+	BI	+	AI	+	F354	AI	+	+	+	+	AI, T360	+	2
HU1016	DU1126	+	+	+	A5	+	+	A351	+	+	B353	A2	+	K21	+	2
HU1017	HU413	A371	+	+	A5	AI	+	A362	AI	+	B353	A2	+	K362	+	2
HU1018	HU413	A371	+	+	A5	AI	+	A363	AI	+	B353	A2	+	K363	+	2
HU1091	DU1438	D1817	+	+	C350	AI	+	A351	A5	B359	B353	+	+	D12, K21	AI	2
HU1148	DU1433	A371	+	+	A5	AI	+	A351	AI or A5	+	B353	A2	G372	K21	+	2
HU1168	DU1519	AI820	+	AI, BI	+	AI	+	A351	AI	+	B353	+	+	K21	+	2
NP187	NP20	+	+	+	+	+	+	A351	A5	+	A351, B353	+	+	K21	+	4
X23	DP53	AI	+	+	+	+	AI	+	+	+	+	+	+	D12	AI	3
XP222	DP1039	A404	+	+	+	+	AI	-392	A5	+	+	+	+	+	+	4

Phenotypes of mutations at the loci indicated are: *acrA*, resistance to 100 µg acriflavine ml⁻¹ or 2% (v/v) methanol; *acrC* and *acrD*, resistance to 100 µg acriflavine ml⁻¹; *arsB*, resistance to 1.5 mg sodium arsenate ml⁻¹; *axe*, ability to grow in axenic media; *bsg*, inability to grow using *Bacillus subtilis* as a food source; *bwnA*, production of brown pigment during development; *cobA*, resistance to 300 µg cobalt chloride ml⁻¹; *cou*, sensitivity to 1.3 mM-coumarin; *cycA*, resistance to 500 µg cycloheximide ml⁻¹; *ebrB*, resistance to 25 µg ethidium bromide ml⁻¹; *frtA*, spore mass at base of fruiting body; *frtB*, distribution of the fruiting bodies in concentric rings; *manA*, α-mannosidase-1 deficient; *sup*, suppression of another mutation, here coumarin-sensitivity due to the *couA351* mutation; *tsg*, temperature-sensitive for growth; *whi*, absence of normal yellow spore pigment. A + denotes the wild-type allele.

* Haploid parents of DU336 are HR7 (Rothman & Alexander, 1975) and HU166 (Williams, 1981), of DU740 are HPS14 (Welker & Deering, 1978) and HU407 (Welker & Williams, 1980c), of DU780 are TS12 (Katz & Sussman, 1972) and HU398 [HU398 is a spontaneous methanol-resistant mutant derived from XP99; for the origin and genotype of XP99 see Ratner & Newell (1978)], of DU1088 are X23 and HU612, of DU1104 are HU611 and X36 (Williams & Newell, 1976), of DU1126 are HU413 and HU694 [HU694 is a spontaneous acriflavine-resistant derivative (*acrC/805*) of γS5 (Welker & Williams, unpublished)], of DU1363 are HU528 and HU960, of DU1433 are HU1018 and NP187, of DU1519 are HU413 and HU1052 [HU1052 is a temperature-sensitive derivative of AX3 (Williams *et al.*, unpublished)], of DPl are TS12 and M28 (Katz & Sussman, 1972), and of DU1438 are NP187 and HU982 [HU982 is a segregant of DU1342 whose parents are TW8 (Welker & Deering, 1978) and HU922; HU922 is a spontaneous ethidium bromide-resistant derivative of HU877 (Welker & Williams, 1980a)].

The genotypes of the following strains have been described previously: HU445 (Welker & Williams, 1980a); it bears the *radC44* mutation in addition to the genotype given; HU154 (Williams & Barrand, 1978), M146 (Free *et al.*, 1976).

† 1, Morrissey *et al.* (1980); 2, this work; 3, Welker & Williams (1980c); 4, Wallace & Newell (1982).

these samples. In some experiments drug-free cultures were prepared as above and then shifted to $28 \pm 1^\circ\text{C}$ to determine the effects of exposure to restrictive temperatures (26.5°C and above) on cell division, cell shape and cell-cell adhesion.

Formation of isogenic diploids. Cells of haploid strains grown overnight in suspension cultures were diluted to about 10^6 ml^{-1} and exposed for 8 h to $10\text{ }\mu\text{g}$ thiabendazole ml^{-1} in PBS in the presence of about 10^{10} *E. coli* B/r ml^{-1} at 21°C as described by Welker & Williams (1980*b*). Survival, as determined by colony-forming ability on SM agar after this treatment, was about 20% and approximately 50% of the colonies were diploid.

Multiple tip formation in drug-treated pseudoplasmodia. Cells were removed from the growing edge of colonies and inoculated on water agar plates which contained 1.5% (w/v) Difco agar and $250\text{ }\mu\text{g}$ dihydrostreptomycin sulphate ml^{-1} . The plates were placed in opaque PVC boxes with lateral light sources at $21 \pm 1^\circ\text{C}$ and the cells were allowed to aggregate to form pseudoplasmodia which migrated towards the light source. Migrating pseudoplasmodia were removed from these plates by lifting them off the agar surface by their slime trails, transferred to inhibitor-containing water agar plates, and returned to the PVC boxes with lateral light sources for further migration. The concentrations of inhibitors used were: coumarin, 1.0–2.0 mM; thiabendazole, 30–50 $\mu\text{g ml}^{-1}$; cambendazole, 80–100 $\mu\text{g ml}^{-1}$; CIPC, 10 $\mu\text{g ml}^{-1}$. Coumarin was added prior to autoclaving the agar; all other inhibitors were added to sterilized molten agar prior to pouring the plates.

Scanning electron microscopy. Scanning electron micrographs were kindly produced for us by Dr M. Claviez and Frau A. Röglin using cells that had been grown and treated with inhibitors in suspension cultures as described above. Samples were fixed for electron microscopy with glutaraldehyde and osmium tetroxide. The fixation technique used was that of Drs G. Gerisch and R. Guggenheim and will be described by them elsewhere.

RESULTS

Isolation of coumarin-sensitive and growth temperature-sensitive mutants

Following MNNG mutagenesis of strain HU526, three coumarin-sensitive mutants (HU609, HU610 and HU612), two temperature-sensitive mutants (HU606 and HU607) and two mutants that were both coumarin-sensitive and temperature-sensitive (HU608 and HU611) were isolated from a total of 1300 colonies screened. Thus, it appears that coumarin-sensitive mutants can be isolated at frequencies similar to those at which temperature-sensitive mutants are found.

Plating efficiencies, relative to that on SM agar, at coumarin concentrations between 0.5 and 1.3 mM were determined for each of the coumarin-sensitive mutants and also their parent, HU526 (Table 2). At 1.3 mM-coumarin, HU526 and other wild-type strains had plating efficiencies of 1.0, while those of coumarin-sensitive mutants ranged from 3×10^{-3} to less than 10^{-6} (Table 2; Welker & Williams, 1980*c*).

Genetic analysis of the coumarin-sensitivity mutations

Four of the new coumarin-sensitivity mutations (*couB352*, *couD355*, *couE353*, *couF354*) are recessive since mutants carrying them formed coumarin-resistant diploids when crossed to the wild-type haploid X23 (Table 3). However, the *couC356* mutation is semi-dominant (Table 3). Complementation tests in which coumarin-sensitive haploid strains were crossed to form heterozygous diploids indicated that the five new coumarin-sensitivity mutations and also the *couA351* mutation each affect a different locus (Table 3). In all cases coumarin-resistant diploid colonies were isolated from SM agar plates containing either 1.0 or 1.3 mM-coumarin after allowing parasexual cell fusion to occur. These experiments were performed at $21 \pm 1^\circ\text{C}$. Thus, complementation of coumarin-sensitivity mutations is an effective method for the selection of heterozygous diploids at permissive growth temperatures.

The *couA351* mutation has been assigned previously to linkage group VII (Welker & Williams, 1980*c*). Parasexual genetic analyses indicated that the newly isolated coumarin-sensitivity mutations map to three of the other six linkage groups. The *couB352* (HU609) mutation was assigned to linkage group I on the basis of its segregation with the *cycA1* (cycloheximide-resistance) mutation (Table 4). The *couC356* (HU608), *couE353* (HU610) and *couF354* (HU611) mutations were assigned to linkage group II on the basis of their segregation relative to mutations at the *whiA* (spore colour), *tsgD* (temperature-sensitivity)

Table 2. Plating efficiencies of wild-type and coumarin-sensitive strains on SM agar plates containing coumarin relative to that on SM agar without coumarin

Strain	Mutation	Relative plating efficiency			
		Coumarin concn (mM):			
		0.5	0.75	1.0	1.3
HU526	+	1.0	1.0	1.2	1.1
HU608	<i>couC356</i>	7.0×10^{-2}	7.3×10^{-5}	$<10^{-6}$	$<10^{-6}$
HU609	<i>couB352</i>	3.4×10^{-3}	3.0×10^{-4}	1.3×10^{-5}	$<10^{-6}$
HU610	<i>couE353</i>	9.8×10^{-1}	8.3×10^{-1}	5.1×10^{-1}	3.0×10^{-3}
HU612	<i>couD355</i>	9.3×10^{-1}	8.8×10^{-1}	2.0×10^{-4}	9.5×10^{-6}
HU876*	<i>couF354</i>	9.2×10^{-1}	6.9×10^{-1}	1.0×10^{-2}	1.1×10^{-3}
NP187	<i>couA351</i>	6.2×10^{-1}	6.6×10^{-3}	3.5×10^{-5}	1.0×10^{-6}

* The original *couF354* coumarin-sensitive mutant, HU611, has a low plating efficiency (about 10^{-4}) on SM agar without coumarin, presumably due to a second mutation not linked to the *couF354* mutation.

All strains in this table had a colony-forming ability in excess of 50% on SM agar. The plating efficiencies presented in this table and earlier work (Welker & Williams, 1980c) were obtained on SM agar prepared using Bacto-peptone from Difco. All others were obtained using media prepared with Oxoid bacteriological peptone which consistently gave higher plating efficiencies.

Table 3. Complementation tests involving coumarin-sensitivity mutations

Diploidy was determined on the basis of spore size and, if applicable, the complementation of recessive mutations other than coumarin-sensitivity mutations. An S denotes non-complementation (plating efficiency less than 0.01 on SM agar containing 1.3 mM-coumarin); an R denotes complementation (plating efficiency close to 1.0 except for diploids heterozygous for the semi-dominant *couC356* mutation where the plating efficiencies were in the range of 0.1 to 0.35 on SM agar containing 1.3 mM-coumarin).

	NP187 (<i>couA351</i>)	HU609 (<i>couB352</i>)	HU608 (<i>couC356</i>)	HU612 (<i>couD355</i>)	HU610 (<i>couE353</i>)	HU611 (<i>couF354</i>)
NP187 (<i>couA351</i>)	S (DU1869)*					
HU609 (<i>couB352</i>)	R (DU950)					
HU608 (<i>couC356</i>)	R (DU932)	R (DU1197)†				
HU612 (<i>couD355</i>)	R (DU953)	R (DU973)	R (DU974)			
HU610 (<i>couE353</i>)	R (DU930)	R (DU1112)	R (DU1111)	R (DU1015)		
HU611 (<i>couF354</i>)	R (DU931)	R (DU984)	R (DU1052)	R (DU1018)	R (DU972)	
X23 (<i>cou</i> ⁺)	R (DU2025)	R (DU1085)	R (DU1089)	R (DU1088)	R (DU1086)	R (DU1118)

* Since all coumarin-sensitivity mutations complemented, the *couA351* mutation was made homozygous by chromosome-doubling using thiabendazole (Welker & Williams, 1980b). The sensitivity of DU1869 constructed in this manner demonstrates that there is no ploidy-specific effect on coumarin-sensitivity, i.e. that homozygous alleles are non-complementing. Further evidence that homozygous coumarin-sensitivity mutations do not complement was obtained with diploids (DU1678 and DU1689) homozygous for the *couA351* mutation which were constructed by standard parasexual techniques based on complementation of *B. subtilis*-sensitivity mutations at 21 °C. DU1678 (HU1016/HU1091) and DU1689 (HU1148/HU1091) were also heterozygous for the *sup-370* and *supG372* mutations which suppress the *couA351* coumarin-sensitivity. Thus, *sup-370* and *supG372* must be recessive.

† The parental haploids of DU1197 were HU608 and HU795 (*couB352*); all other diploids were constructed by fusion of the haploids shown.

and/or *acrA* (methanol-resistance) loci (Table 4). The *couD355* (HU612) mutation was assigned to linkage group III on the basis of its segregation with the *bsgA5* (*B. subtilis*-sensitivity) mutation (Table 4). In all cases, the coumarin-sensitivity mutations were found to segregate independently of markers on the five linkage groups to which they were not assigned (Table 4).

Table 4. Genotypes of haploid segregants derived from diploids heterozygous for coumarin-sensitivity mutations

Diploid (Parental haploids)	Locus of interest	Linkage group*					
		I	II	III	IV	VI	VII
DU1085 (HU609, X23)			<i>+ /whiA1</i> <i>+ /tsgD12</i>				
	<i>cycA1</i> /+		<i>+ /acrA1</i>	<i>bsgA5</i> /+	<i>bwnA1</i> /+	<i>manA2</i> /+	<i>+ /cobA1</i>
	<i>couB352</i> +	16 0 0 32	6 10 4 28	8 8 24 8	4 12 10 22	15 1 2 30	10 6 17 15
DU1089 (HU608, X23)			<i>+ /whiA1</i> <i>+ /tsgD12</i> <i>+ /acrA1</i>	<i>bsgA5</i> /+ <i>tsgR358</i> /+			
	<i>cycA1</i> /+				<i>bwnA1</i> /+	<i>manA2</i> /+	<i>+ /cobA1</i>
	<i>couC356</i> +	24 23 13 28	47 0 0 41	24 23 9 32	10 37 9 32	13 34 10 31	32 15 33 8
DU1088 (HU612, X23)			<i>+ /whiA1</i> <i>+ /tsgD12</i> <i>+ /acrA1</i>	<i>bsgA5</i> /+			
	<i>cycA1</i> /+				<i>bwnA1</i> /+	<i>manA2</i> /+	<i>+ /cobA1</i>
	<i>couD355</i> +	8 24 17 31	14 18 19 29	32 0 0 48	9 23 10 38	13 19 17 31	28 4 32 16
DU1205† (HU799, HL33)			<i>acrA1808</i> /+ <i>+ /tsgP500</i>	<i>bsgA5</i> /+ <i>+ /whiB500</i>	<i>bwnA1</i> /+	<i>manA2</i> /+	<i>+ /bsgB500</i>
	<i>cycA1</i> /+						
	<i>couE353</i> +	7 8 17 3	15 0 0 20	8 7 18 2	7 8 7 13	3 12 5 15	0 7 1 1
DU1309 (HU876, HU215)			<i>tsgT360</i> /+ <i>+ /whiA1</i> <i>+ /tsgD12</i> <i>+ /arsB353</i>	<i>tsgA1</i> /+ <i>+ /acrC4</i> <i>axeB1/axeB1</i>			
	<i>cycA1</i> /+				<i>bwnA1</i> /+		
	<i>couF354</i> +	6 2 3 5	8 0 0 8	8 0 1 7	7 1 3 5		
DU1310‡ (HU876, HU528)			<i>tsgT360</i> /+ <i>+ /whiA1</i> <i>+ /tsgD12</i>	<i>+ /bsgA5</i> <i>tsgA1</i> /+ <i>axeB1</i> /+			
	<i>cycA1/cycA1</i> or <i>A5</i>				<i>bwnA1</i> /+	<i>+ /manA2</i>	<i>+ /cobA1</i>
	<i>couF354</i> +		14 0 0 45	3 11 12 33	9 5 28 17	0 14 0 45	10 4 29 16

* Linkage group V remains to be established genetically.

† The presence of the *bsgB500* mutation was scored only in segregants that did not carry the *bsgA5* mutation.

‡ All segregants of DU1310 were cycloheximide-resistant. DU1310 was heterozygous at the *manA* locus but only segregants carrying the *manA2* allele were obtained. It is possible that the chromosome carrying the *manA*⁺ allele may have acquired a recessive-lethal mutation.

No evidence for the presence of suppressor mutations like *sup-370* or *supG372* was found with these diploids.

Genetic analysis of the temperature-sensitivity mutations

Since three of the six coumarin-sensitive mutants isolated in *D. discoideum* (NP187, HU608 and HU611) acquired a growth temperature-sensitivity in the same mutagenesis that produced the coumarin-sensitivity, it was important to attempt to determine whether or not the coumarin-sensitive and temperature-sensitive phenotypes were the pleiotropic effect of a single genetic lesion. With strain NP187 we have shown previously that the coumarin-sensitivity and temperature-sensitivity are both on linkage group VII (Welker & Williams,

Table 5. Genotypes of haploid segregants derived from diploids heterozygous for temperature-sensitivity mutations

Diploid (Parental haploids)	Locus of interest	Linkage group*					
		I	II	III	IV	VI	VII
DU1087 (HU606, X23)			<i>+/whiA1</i> <i>+/tsgD12</i> <i>+/acrA1</i>	<i>bsgA5/+</i>	<i>bwnA1/+</i>	<i>manA2/+</i>	<i>+/cobA1</i>
	<i>tsgQ356</i> +	<i>cycA1/+</i> 15 0 0 13	15 0 13 0	15 0 2 11	11 4 4 9	15 0 0 13	10 5 7 6
DU1261 (HU881, HU215)			<i>+/whiA1</i> <i>+/arsB353</i> <i>+/tsgD12</i> <i>acrA1812/+</i>	<i>bsgA5/+</i> <i>+/acrC4</i> <i>+/axeB1</i>	<i>bwnA1/+</i>	<i>manA2/+</i>	
	<i>tsgQ356</i> +	<i>cycA1/+</i> 4 0 0 10	4 0 10 0	1 3 8 2	0 4 1 9	0 4 2 8	
DU1089 (HU608, X23)			<i>couC356/+</i> <i>+/whiA1</i> <i>+/tsgD12</i> <i>+/acrA1</i>	<i>bsgA5/+</i>	<i>bwnA1/+</i>	<i>manA2/+</i>	<i>+/cobA1</i>
	<i>tsgR358</i> +	<i>cycA1/+</i> 24 0 0 23	24 0 23 0	24 0 0 23	7 17 3 20	13 11 0 23	24 0 8 15
DU1271 (HU608, HU215)			<i>couC356/+</i> <i>+/whiA1</i> <i>+/arsB353</i> <i>+/tsgD12</i>	<i>bsgA5/+</i> <i>+/acrC4</i> <i>+/axeB1</i>	<i>bwnA1/+</i>	<i>manA2/+</i>	
	<i>tsgR358</i> +	<i>cycA1/+</i> 8 5 3 1	13 0 4 0	13 0 0 4	1 12 1 3	12 1 0 4	

* Linkage group V remains to be established genetically.

Only data obtained with yellow, *tsgD*⁺ segregants is presented. White, temperature-sensitive (*tsgD12*) segregants were obtained in all segregations but were excluded from the data presented since the *tsgQ356* and *tsgR358* mutations would be masked by the presence of the *tsgD12* mutation.

Cosegregation of linkage groups I and III (and VI) occurred in the segregants of DU1087 and DU1089. Similar linkage associations have been seen previously in *D. discoideum* (Welker & Williams, 1980c).

1980c); thus, they may be the pleiotropic effects of a single mutation. Since the coumarin-sensitivity of strain HU611 (*couF354*) mapped to linkage group II (Table 4), a diploid (DU1363) which was heterozygous for coumarin-sensitivity, temperature-sensitivity and methanol-resistance (*couF354 tsgT360 acrA1814/couF*⁺ *tsgT*⁺ *acrA*⁺) was constructed to test for linkage of the coumarin-sensitivity and temperature-sensitivity on the basis of mitotic recombination (or non-disjunction). Four independently isolated methanol-resistant diploids selected from 8×10^5 cells of DU1363 were coumarin-sensitive and temperature-sensitive (*couF354 tsgT360 acrA1814/couF354 tsgT360 acrA1814*). Thus, both the coumarin-sensitivity (*couF354*) and temperature-sensitivity (*tsgT360*) of strain HU611 and its derivatives map to linkage group II and could be the pleiotropic effects of a single mutation. The *tsgR358* mutation of strain HU608 was assigned to linkage group III on the basis of its segregation with the *bsgA5* mutation and segregation independent of markers on linkage groups I, II, IV, VI and VII (Table 5). However, strains that carried the linkage group II bearing the *couC356* mutation, but no known temperature-sensitivity mutation, had plating efficiencies of 0.006 to 0.3 at 26.5 °C. Thus, the *tsgR358* and *couC356* mutations certainly

Table 6. *Plating efficiencies at 26.5 °C and on SM agar containing coumarin of strains bearing the *tsgK21/couA351* mutation(s) and of revertants derived from them on the basis of temperature-resistance*

The revertants are of certain independent origin except for the pair HU1017 and HU1018 and the pair HU1166 and HU1167. These pairs of revertants are probably of independent origin also since they are phenotypically different. The plating efficiencies are given as the ratio of colony-forming ability under the restrictive condition relative to that at 21 °C on SM agar. Two of these revertants, HU1195 and HU1196, are also altered in development, having smaller sori and thinner stalks than their parent NP187.

Strain	Parent	Relative plating efficiency		
		At 26.5 °C	With 1.0 mM-coumarin	With 1.3 mM-coumarin
HU413	—	$<10^{-5}$	3.0×10^{-2}	$<10^{-5}$
HU1168	—	$<10^{-5}$	1.5×10^{-3}	$<10^{-5}$
NP187	—	$<10^{-5}$	0.4	5×10^{-4}
HU1017	HU413	0.4	1.1	0.2
HU1018	HU413	0.7	1.1	1.0
HU1202	HU413	0.5	0.8	6×10^{-3}
HU1203	HU413	3×10^{-2}	0.8	4×10^{-3}
HU1200	HU1168	0.1	1.0	0.3
HU1166	NP187	1.0	1.0	1.0
HU1167	NP187	0.1	0.7	0.3
HU1193	NP187	0.3	1.1	0.7
HU1195	NP187	0.4	1.0	0.5
HU1196	NP187	0.5	0.9	0.7
HU1197	NP187	0.1	0.9	0.4
HU1198	NP187	0.1	1.0	0.7
HU1199	NP187	0.1	1.1	0.6

affect different loci but there may also be a slight temperature-sensitivity associated with the *couC356* mutation.

The *tsgQ356* mutation of strain HU606, which was isolated at the same time as the coumarin-sensitive mutants, was assigned to linkage group I on the basis of its segregation with the *cycA1* mutation and segregation independent of markers on linkage groups II, III, IV, VI and VII (Table 5). The *tsgM357* mutation of strain HU607 was mapped to linkage group VII (Welker & Williams, 1980c).

*Co-reversion of the temperature-sensitivity and coumarin-sensitivity in temperature-resistant revertants of strains that carry the *tsgK21/couA351* mutation(s)*

Evidence supporting the hypothesis that the *tsgK21* temperature-sensitivity and *couA351* coumarin-sensitivity are the pleiotropic effects of a single mutation was obtained with putative revertants selected on the basis of temperature-resistance from strains NP187, HU413 and HU1168. All eleven independently isolated temperature-resistant derivatives (seven from NP187, one from HU1168 and three from HU413) were more resistant to coumarin than their parents (Table 6). Most of these strains clearly did not have wild-type phenotypes, since they had lower colony-forming ability at 26.5 °C and on media containing coumarin than at 21 °C on SM agar (Table 6). Genetic analysis of putative revertants of strain HU413 (HU1017 and HU1018, which may not be of independent origin) gave results consistent with either a reversion at the *tsgK/couA* locus or with another mutation on linkage group VII that suppresses both the growth temperature-sensitivity and coumarin-sensitivity (data not shown). If such a suppressor mutation exists in HU1017 or HU1018, it must be dominant, since heterozygous diploids formed by fusion of NP187 with HU1017 or HU1018 were

selected at the restrictive temperature (26.5 °C). These diploids, DU1489 and DU1433, respectively, which arose at a lower than normal frequency, had plating efficiencies of only about 0.01 at 26.5 °C and 0.20 on SM agar containing 1.3 mM-coumarin. The low plating efficiencies of these diploids are consistent with the partial reversion of the temperature-sensitivity and coumarin-sensitivity seen in the haploid revertants. In comparison, homozygous *tsgK21/couA351* diploids formed by chromosome doubling of HU413 (DU1850) and NP187 (DU1869) had plating efficiencies at 26.5 °C of less than 10^{-6} and on SM agar containing 1.3 mM-coumarin of about 0.003 and 0.01, respectively. Plating efficiencies for HU413 and NP187, determined concurrently with those of DU1850 and DU1869, were similar to those of DU1850 and DU1869, respectively.

The coumarin-sensitivity, but not the growth temperature-sensitivity, of derivatives of strain NP187 can certainly be suppressed by mutations at loci other than *couA*. We have evidence for at least two such mutations (*sup-370* and *supG372*) which do not map on linkage group VII. Strains carrying the *tsgK21* mutation in the presence of either of these suppressor mutations remain temperature-sensitive. Strains HU1016 (*sup-370 tsgK21/couA351*) and HU1148 (*supG372 tsgK21/couA351*) had plating efficiencies on SM agar at 26.5 °C of less than 10^{-6} but of about 1.0 on SM agar containing 1.3 mM-coumarin. Both the *sup-370* and *supG372* mutations are recessive since diploids (DU1678 and DU1689, respectively) constructed by fusion of HU1016 and HU1148 with strain HU1091 (*couA351/tsgK21*) were temperature-sensitive and had plating efficiencies on SM agar-containing 1.3 mM-coumarin of about 0.002 and 0.01, respectively. Many of the colonies which did appear on plates containing 1.3 mM-coumarin with these diploids were actually haploid segregants that bore the suppressor mutation.

Coumarin-resistance may arise from second-site mutations that affect membrane permeability but which leave the original mutation unaffected, while selection for temperature-resistance may be more stringent and lead to the isolation only of strains able to grow both at the restrictive temperature and in the presence of coumarin. The *supG372* mutation probably does affect the permeability of the cell membrane since, in addition to increased resistance to coumarin, strains bearing this mutation have decreased resistance to cycloheximide and acriflavine. The *supG372* mutation was discovered in the genetic analysis of strain HU1018, a temperature-resistant revertant of strain HU413. It must have arisen during the isolation of this strain since the parent of HU1018 (HU413) does not carry the *supG372* mutation. We suggested previously that the *tsgK21* and *couA351* mutation(s) affected different loci (Welker & Williams, 1980c) on the basis of strains which were coumarin-resistant but temperature-sensitive. These findings can be explained by the presence of suppressor mutations like *supG372* rather than reversion of a coumarin-sensitivity mutation alone.

Inhibition of cell division by coumarin

We have shown previously that wild-type cells of *D. discoideum* have little or no loss of colony-forming ability on SM media containing 1.0 or 1.3 mM-coumarin (Welker & Williams, 1980c; see also Table 2). However, we observed that the growth rate was decreased on media containing coumarin; specifically, colonies appeared later and expanded more slowly in the presence of coumarin. The effect of coumarin on wild-type and coumarin-sensitive strains was investigated further in suspension culture in the presence of pregrown bacteria. This procedure eliminates any effects which coumarin might have on bacterial growth and allows changes in cell number to be easily determined.

The doubling times of cells of the wild-type strain, HU526, were found to increase with increasing concentrations of coumarin between 1.0 and 2.5 mM. At concentrations of 1.0 and 1.3 mM-coumarin the doubling times were about 4.5 and 5.5 h, respectively, while that of the untreated control culture was less than 4.0 h. Concentrations of 2.5 mM, or more,

Fig. 1. Growth curves of the wild-type coumarin-resistant haploid HU526 (a) and of the *couA351* coumarin-sensitive haploid HU413 (b) in PBS in the presence of about 10^{10} *E. coli* B/r ml $^{-1}$ under illumination with rotation at 180 rev. min $^{-1}$. Cells were treated at 21 ± 1 °C with no coumarin (○), 1.0 mM-coumarin (●), 1.3 mM-coumarin (□), 1.6 mM-coumarin (■) or 2.5 mM-coumarin (▲), or at 28 ± 1 °C (△).

Table 7. Cell-cell adhesion induced by different treatments

Cell-cell adhesion was assayed in PBS in the presence of about 10^{10} *E. coli* B/r ml $^{-1}$. Cell suspensions (5 ml) were rotated under illumination at 180 rev. min $^{-1}$. In all cases, cell division was completely inhibited by these treatments and the treated cells became spherical. Untreated control cells prepared and handled similarly were irregular in shape and continued to divide. Less than 5% of control cells were in contact prior to the stationary phase (18–24 h). Adhesion was observed in some experiments at lower coumarin concentrations than those given here; with lower coumarin concentrations longer times were required before adhesion was observed and fewer cells were involved. At higher concentrations adhesion was seen earlier and a greater percentage of the cells were involved. The number of cells in any one group varied from two to ten or more but pairs were the most common type seen. The data presented are from representative experiments. The times of assay varied from strain to strain within the stated limits. Cell-cell adhesion was not seen with HU609 (*couB352*); this strain lysed when exposed to 1.6 mM-coumarin.

Strain	Treatment	Cell-cell adhesion (percentage of cells in contact)			
		Duration of treatment:			
		2–3 h	4.5–6 h	8.5–10 h	23–24.5 h
HU526 (<i>cou</i> ⁺)	3.0 mM-coumarin	64	—	79	—
HU526 (<i>cou</i> ⁺)	50 µg thiabendazole ml $^{-1}$	45	33	—	—
HU526 (<i>cou</i> ⁺)	100 µg cambendazole ml $^{-1}$	69	—	—	—
HU526 (<i>cou</i> ⁺)	25 µg CIPC ml $^{-1}$	78	79	73	—
HU413 (<i>couA351</i>)	1.6 mM-coumarin	3	10	89	66
HU413 (<i>couA351</i>)	28 °C	25	70	>95	46
HU608 (<i>couC356</i>)	1.0 mM-coumarin	45	45	20	—
HU833 (<i>couD355</i>)	2.5 mM-coumarin	57	64	78	89
HU610 (<i>couE353</i>)	1.6 mM-coumarin	<5	30	65	50
HU970 (<i>couF354</i>)	1.6 mM-coumarin	<5	56	52	31
HU970 (<i>couF354</i>)	28 °C	<5	<5	36	70

completely inhibited cell division (Fig. 1a). Similar effects were seen with all strains carrying mutations that lead to coumarin-sensitivity. The major difference between these mutants and wild-type strains was that cell division of the coumarin-sensitive strains was inhibited at lower

Fig. 2. Scanning electron micrographs of cells of wild-type strain HU526: untreated control (a, b); treated with 5 mM-coumarin for 3 h (c, d); and treated with 125 μg thiabendazole ml^{-1} for 3 h (e, f). The diameters of cells shown are approximately 7 μm .

concentrations of coumarin. For example, HU413, a *couA351* strain, has a doubling time of about 13 h in the presence of 1.0 mM-coumarin and cell division was completely inhibited at 1.6 mM-coumarin, while untreated cultures grew at a rate similar to that of untreated cultures of the wild-type strain HU526 (Fig. 1 b).

Cell-cell adhesion of vegetative cells in the presence of coumarin and mitotic inhibitors

At coumarin concentrations which completely inhibited cell division, the cells appeared more refractile and spherical than untreated cells when observed by light microscopy. In addition, the cells of wild-type strains and of most coumarin-sensitive mutants adhered to each other in the presence of coumarin at concentrations that completely inhibited cell division (Table 7). Groups of two to more than ten cells were seen but most cells were in pairs. The extent of cell-cell adhesion (percentage of cells involved, number of cells per group, rate of group formation etc.) was dependent on drug concentration. The drug concentrations used to obtain the data presented in Table 7 were chosen as the lowest to give reproducible cell-cell adhesion within about 5 h. Scanning electron microscopy of coumarin-treated cells of the wild-type strain HU526 showed that the coumarin-treated cells were spherical and lacked the numerous filopodia seen on untreated cells (Fig. 2). EDTA at a concentration of 1 mM completely inhibited this cell-cell adhesion. Thus, contact sites B, which are known to exist on vegetative cells and whose interaction is EDTA-sensitive (Gerisch, 1961), may be involved in this phenomenon. The major difference between the coumarin-sensitive mutants

and wild-type strains was that with the mutants this cell-cell adhesion occurred at lower coumarin concentrations (Table 7).

The only coumarin-sensitive mutant in which cell-cell adhesion was not induced was the *couB352* strain, HU609. Instead, the cells of HU609 lysed in the presence of coumarin at concentrations of 1.6 mM or more. HU609 was the only mutant in which this phenotype was encountered. Lysis was also seen on treatment of the wild-type strain HU526 with cambendazole (80–200 $\mu\text{g ml}^{-1}$) and CIPC (50 $\mu\text{g ml}^{-1}$) but not with coumarin (up to 5 mM).

Similar cell-cell adhesion occurred when the wild-type strain HU526 was treated with thiabendazole (50–200 $\mu\text{g ml}^{-1}$), cambendazole (80–200 $\mu\text{g ml}^{-1}$) or CIPC (25 $\mu\text{g ml}^{-1}$). In particular, thiabendazole-treated cells appeared identical to coumarin-treated cells in the scanning electron microscope (Fig. 2). At lower concentrations (10, 50 and 5 $\mu\text{g ml}^{-1}$, respectively) all three compounds have been shown to induce metaphase arrest in *D. discoideum* growing on bacteria under conditions similar to those used here (Welker & Williams, 1980*b*; unpublished results for CIPC-induced metaphase arrest). In addition, thiabendazole (2 $\mu\text{g ml}^{-1}$), cambendazole (10 $\mu\text{g ml}^{-1}$) and CIPC (1 $\mu\text{g ml}^{-1}$) all induce haploidization of diploid cells of *D. discoideum* (Welker & Williams, 1980*b*; unpublished results for CIPC-induced haploidization).

Cell-cell adhesion at high temperature

Further evidence that the growth temperature-sensitivity and coumarin-sensitivity of strains carrying *tsgK21/couA351* mutation(s) were due to a single mutation was obtained from suspension cultures incubated at 28 °C (slightly above the normally used restrictive temperature, 26.5 °C) in the absence of coumarin. Under these conditions cells of strain HU413 (*tsgK21/couA351*) formed cell-cell contacts that appeared identical to those formed at 21 °C in the presence of coumarin (Table 7). Cells of the coumarin-sensitive, temperature-sensitive strain HU970 (*tsgT360/couF354*) also formed contacts at 28 °C (Table 7); thus, the two phenotypes may be associated with a single mutation in this strain also. Such temperature-induced cell-cell adhesion was not observed in either the wild-type strain HU526 or haploids that bore the following temperature-sensitivity mutations: *tsgA1* (HU445), *tsgD12* (TS12, HU154), *tsgE13* (HU154), *tsgH353* (M146) and *tsgR358* (HU608). Nor was cell-cell adhesion induced at 28 °C in haploids that carried the *couB352* (HU609), *couC356* (HU608), *couD355* (HU833) or *couE353* (HU610) mutations. Thus, the temperature-induced cell-cell adhesion of haploids which carry the *tsgK21/couA351* or *tsgT360/couF354* mutations appears to be specific to these strains.

Multiple tip formation in pseudoplasmodia treated with coumarin, thiabendazole or cambendazole

The inhibitors colchicine and CIPC have previously been shown to induce multiple tip formation in pseudoplasmodia of *D. discoideum* (O'Day & Durston, 1978; White *et al.*, 1981). Coumarin (1.0–2.0 mM), thiabendazole (30–50 $\mu\text{g ml}^{-1}$) and cambendazole (80–100 $\mu\text{g ml}^{-1}$) all induced multiple tip formation in the wild-type strain HU526 as did CIPC (10 $\mu\text{g ml}^{-1}$).

Lack of evidence for metaphase arrest or haploidization by coumarin

While coumarin induces some effects like those caused by the mitotic inhibitors thiabendazole, cambendazole, CIPC and colchicine it does not appear to induce metaphase arrest in bacterially grown cells of *D. discoideum* or to induce haploidization. In cells of strain HU526 treated with either 3 or 5 mM-coumarin for 3 h no metaphase figures were seen, while control cultures had about 2% metaphase figures and a culture treated with 1 mM-coumarin had 1.5% metaphase figures (cell division of HU526 is only slightly inhibited at this concentration; Fig. 1*a*). With HU413 (*couA351*), untreated control cultures had about 2%

metaphase figures, but no metaphase figures were seen after treatment for 3 h with 1.0, 1.3, 1.6, 1.8 or 2.0 mM-coumarin.

Diploid cells of DP1 did not sector haploids on SM agar containing 1.3 mM-coumarin. Thus, coumarin does not appear to be a haploidizing agent like benlate, thiabendazole, cambendazole or CIPC. The fact that heterozygous diploids can be selected from mixed populations of coumarin-sensitive parental haploids on media containing 1.3 mM-coumarin is further evidence that coumarin does not induce haploidization (Table 3).

DISCUSSION

Six coumarin-sensitivity mutations are now known in *D. discoideum*. With the exception of the *couC356* mutation which is semi-dominant, these mutations are recessive. Two (*couA351* and *couF354*) are linked to temperature-sensitivities that may be the pleiotropic effects of the coumarin-sensitivity mutations. Exposure to high temperature (28 °C) induced cell-cell adhesion in strains bearing either the *couA351* or *couF354* mutation similar to that seen on exposure to coumarin. This phenotype was not observed at 28 °C with strains other than those carrying the *couA351* or *couF354* mutations. Further evidence for the pleiotropy of the coumarin-sensitivity and temperature-sensitivity of strains carrying the *couA351/tsgK21* mutation(s) was obtained with revertants selected on the basis of temperature-resistance. All eleven independently isolated revertants were more coumarin-resistant than their parents. The isolation of temperature-resistant strains which carry a reversion of or suppressor of the *tsgK21* mutation and which are also coumarin-resistant strongly implies that the growth temperature-sensitivity and coumarin-sensitivity of strains carrying the *couA351/tsgK21* mutation(s) are closely related properties. The simplest interpretation of these results is that the coumarin-sensitivity and growth temperature-sensitivity of strains that carry the *couA351/tsgK21* mutation(s) are the pleiotropic effects of a single mutation on linkage group VII. Similar effects have been observed with certain mutations in the gene for β -tubulin of *Aspergillus nidulans* which lead simultaneously to benomyl-resistance and temperature-sensitivity (Sheir-Neiss *et al.*, 1978).

Inconsistent with our interpretation of pleiotropy at the *couA/tsgK* locus are the results of Wallace & Newell (1982) in which the temperature-sensitivity and coumarin-sensitivity of strains carrying the *tsgK21* and *couA351* mutation(s) were apparently separated by mitotic recombination. In order to test whether or not the *couA351* and *tsgK21* mutations were separated we constructed a diploid between the *couA351 tsgK21* haploid HU413 and XP222, a coumarin-sensitive temperature-resistant (*couA351?* *tsgK*⁺) haploid segregant of one of the putative mitotic recombinants, which was kindly sent to us by J. S. Wallace and P. C. Newell. If XP222 carried the *couA351* allele, we would not expect to be able to isolate a diploid on the basis of complementation of coumarin-sensitivity mutations between XP222 and a strain containing the *couA351* mutation. However, we were able to isolate diploids between HU413 and XP222 by this method. Wallace & Newell (1982) have confirmed this result by isolating diploids between NP187 and XP222. Our diploid, DU1787, has a plating efficiency of greater than 0.5 on SM agar containing 1.3 mM-coumarin, though it does grow more slowly than most other diploids heterozygous for coumarin-sensitivity mutation(s). The diploid of Wallace & Newell (1982) is similar in phenotype to DU1787. They interpreted their data as indicating that XP222 and NP187 carried the same coumarin-sensitivity mutation. We believe this conclusion is unwarranted. We conclude that XP222 carries a coumarin-sensitivity mutation (*cou-392*) of unknown origin which is not identical to the *couA351* mutation. Unfortunately, other haploid segregants of putative recombinants in the presumed *couA-tsgK* interval were not kept by Wallace & Newell so it is not possible at the present time to investigate the properties of such strains. Until this is done we feel that the genetic separation of the *couA351* and *tsgK21* mutations has not been conclusively established.

There are now four classes of compounds all of which appear to affect the microtubular system of *D. discoideum*. These are colchicine (Cappuccinelli & Ashworth, 1976; O'Day & Durston, 1978; Zada-Hames, 1977), benzimidazole derivatives including nocodazole (Cappuccinelli *et al.*, 1979), thiabendazole, cambendazole and benomyl (Welker & Williams, 1980b; Williams & Barrand, 1978), CIPC and related compounds (White *et al.*, 1981; Cappuccinelli & Ashworth, 1976; this report), and coumarin. Treatment of vegetative cells with coumarin, thiabendazole or CIPC inhibited division, resulted in a loss of filopodia, led to a spherical shape and induced EDTA-sensitive cell-cell adhesion that could reflect the patching of cell surface molecules such as contact sites B. Coumarin, thiabendazole and cambendazole induced multiple tip formation in pseudoplasmodia as did colchicine (O'Day & Durston, 1978) and CIPC (White *et al.*, 1981).

In addition to these effects on cell shape and pseudoplasmodial organization, colchicine, the benzimidazole derivatives and CIPC inhibit the function of the mitotic spindle. Benzimidazole derivatives and CIPC induced metaphase arrest and haploidization at low doses in *D. discoideum* (Welker & Williams, 1980b; White *et al.*, 1981; this report), although benomyl was ineffective at the induction of metaphase arrest in *D. discoideum* but not in another slime mould, *Polysphondylium pallidum* (Williams, 1980). Colchicine was relatively ineffective at inducing metaphase arrest in *D. discoideum* (Zada-Hames, 1977) and *P. pallidum* (Williams, 1980). In *D. discoideum*, coumarin is neither a haploidizing agent nor a mitotic poison; therefore it may not affect the mitotic spindle.

Similar effects on cell shape and cell division to those found in *D. discoideum* have been seen with coumarin-treated cells of *Euglena gracilis* (Fasulo *et al.*, 1979; Vannini *et al.*, 1977). In *E. gracilis*, coumarin induced a lengthening of the G2 phase of the cell cycle but cytoplasmic and flagellar microtubules remained visible (Fasulo *et al.*, 1979). Microtubules also remained visible in coumarin-treated protoplasts of the fresh water alga *Mougeotia* (Marchant, 1979) and in cells of pine seedlings (Itoh, 1976). Fasulo *et al.* (1979) suggest that coumarin may act by disturbing microtubule organizing centres. Such an activity is not inconsistent with our observations in *D. discoideum*.

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