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Genetics and biochemistry of 5-Bromodeoxyuridine resistance in *Physarum polycephalum*

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SUMMARY

5-Bromodeoxyuridine (BrdU)-resistant mutants of *Physarum polycephalum* were isolated as colonies of myxamoebae growing on BrdU-substituted bacteria after exposure to long-wave ultraviolet light (UV). Twenty-four such mutants were studied. They all show Mendelian segregation in crosses with wild type. Plasmodia constructed from mutant amoebae were all deficient in deoxythymidine incorporation. Extracts made from selected plasmodia showed that all except one had low thymidine kinase activity.

Genetical and biochemical complementation studies revealed two complementation groups: 23 mutants, *bur* A, had low thymidine kinase while 1 mutant, *bur* B, had normal thymidine kinase levels.

1. INTRODUCTION

5-Bromodeoxyuridine (BrdU) is an analogue of thymidine (TdR) which is readily incorporated into the DNA of most cells and viruses. When inserted it may retard cell growth and makes the DNA light-sensitive. Thus, long-wave ultraviolet (UV) irradiation (310–320 nm) specifically causes irreparable damage in a genome partially substituted with BrdU for TdR. This has been used to enrich for specific mutant classes, by imposing 'non-permissive' conditions on a cell population growing in the presence of BrdU. 'Non-permissive' conditions can be high temperature if heat-sensitive mutants are sought or the absence of a nutrient factor if auxotrophic mutants are sought. Here, wild-type cells continue to grow, and are killed or retarded by the presence of BrdU in their DNA. Mutant cells, unable to grow in non-permissive conditions, will be spared and can be recovered (Carl, 1970; Kao & Puck, 1969).

However, mutants resistant to BrdU (obtained with or without concomitant UV irradiation, depending on the cell type involved) which are still able to grow in the presence of BrdU have been found in a number of cell types. Mechanisms for this resistance involve loss of thymidine kinase, necessary for the utilization of both TdR and BrdU, TdR and BrdU uptake defects, as well as other functions not understood at present (Hiraga, Igarashi & Yura, 1967;

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Chace & Hall, 1973; Freed & Mezger-Freed, 1973; Roufa, Sadow & Caskey, 1973; Bright & Northcote, 1974; Ohyama, 1974).

When BrdU-suicide enrichment for heat-sensitive mutants was applied to *Physarum polycephalum* myxamoebae, BrdU-resistant mutants arose (Haugli & Dove, 1972). Here, BrdU resistance means the ability of cells to survive longwave UV irradiation after growth on BrdU-substituted bacteria.

In the present work, 24 BrdU-resistant mutants have been studied, to elucidate the genetical and biochemical basis of such resistance in *Physarum*.

2. MATERIALS AND METHODS

(i) *Strains and loci*

Strain	Genotype		Reference
TU4	mt_1	—	Formerly RSD4 and RSD8
TU8	mt_2	—	(Haugli, 1971)
TU52	mt_1	*bur* A52	(Haugli, 1971)
TU291	Wild-type plasmodium		TU4 × TU8 (Haugli, 1971)
LU648	mt_1	*fus*AI *leu*-1+	All strains are *fus* B1. The origin of these strains has been described previously (Cooke, 1974)
LU657	mt_1	*fus*A2 *leu*-1+	
LU688	mt_2	*fus*A1 *leu*-1+	
LU850	mt_h	*fus*A1 *leu*-1−	
LU853	mt_1	*fus*A2 *leu*-1−	
CL	mt_h	*fus*A2 *leu*-1+	Cooke & Dee (1975)
a	mt_1	*fus*A1	Dee (1966)

(The designations TU and LU refer to Tromsø University, and Leicester University stock numbers.)

The LU strains listed above are all strains isogenic with CL (Cooke & Dee, 1975).

In experimental set I mutations were induced in amoebae of strain TU4 and the mutants crossed with TU8. (In one instance TU4 and TU8 were replaced by LU688 and LU648.)

In experimental set II mutations were induced in LU850d amoebae, which is a derivative of LU850 with delayed plasmodium formation.

Explanation of loci designations used: *mt*, mating type, with alleles mt_1 and mt_2 in the heterothallic strains used here (Dee, 1966). In these strains diploid plasmodia are formed after mating of haploid amoebae of different mating type. Strains of genotype mt_h are apogamic, haploid amoebae. These differentiate into haploid plasmodia within single amoebal clones (Wheals, 1970; Cooke & Dee, 1974; Anderson, Cooke & Dee, 1976). *fus*A and *fus*B are plasmodial fusion types (Cooke & Dee, 1974).

(ii) *Culture techniques*

Myxamoebae were grown on 0·05% liver infusion (Oxoid) plates and fed live or formaldehyde-inactivated *E. coli* (Haugli & Dove, 1972).

Crossing of heterothallic strains, plasmodium formation by mt_h strains, and crossing heterothallic and mt_h strains was achieved by inoculating amoebae in a puddle of live bacteria on plates with 1/10 strength plasmodial growth medium. The identification of crossed plasmodia resulting from mixtures of mt_h and hetero-

thallic amoebae, using the plasmodial fusion system, has been described previously (Cooke, 1974; Cooke & Dee, 1975).

Microplasmodia were grown in baffled flasks in 15 ml of growth medium on a gyratory shaker (160 rev/min). The semi-defined medium used was that described by Daniel and Baldwin (1969) or that described by Dee & Poulter (1970). Sporulation was achieved by exposing plasmodia to daylight after exhaustion of the nutrient medium.

(iii) *Mutagenesis and screening procedures*

Nitrosoguanidine (NG) was used in some experiments in attempts to improve mutant yield. There is at present no clear indication that NG does increase the frequency of mutation to BrdU-resistance in *P. polycephalum*. When used, growing cells were suspended in 0·05 M phosphate buffer, pH 7, containing NG at 40 μg/ml for 60 min. Cells were then allowed to recover on liver infusion plates before plating and screening for mutants.

Whether mutagenized or not, the screening was done by growing the myxamoebae on BrdU-substituted formaldehyde inactivated bacteria on liver infusion plates (Haugli & Dove, 1972). After about two cell-division cycles the cells were exposed daily to irradiation from two Westinghouse FS20 sunlamps (wavelength maximum at 320 nm) from a distance of 30 cm through a shield of two plastic Petri-dish lids. The irradiation dose sufficient to prevent growth of wild type under these conditions was about 20 min. Sometimes larger doses or combinations of larger and smaller doses were used. Colonies (plaques) arising from 4 to 10 days after initiation of the selective regime were picked and retested.

(iv) In vivo *assay for thymidine incorporation*

In experimental set I, synchronous plasmodia 30 min past metaphase II or III (at peak activity of thymidine kinase, Hildebrandt & Sauer, 1973) were used to measure uptake of ^{3}[H]thymidine into DNA. Circular, 1 cm^{2} disks of plasmodia supported on Millipore membranes were cut out and incubated at 28 °C on a 25 μl droplet of the regular growth medium containing 0·5 μCi/ml of ^{3}[H]thymidine at specific activity 19 Ci/mmole (Amersham, code TRK-120). Incubations were terminated by immersing the plasmodia in ice-cold 5% TCA in acetone. The material was extracted twice with ice-cold 5% TCA-acetone and twice with ice-cold 0·25 M PCA and then dissolved in 1 ml of 0·4 N-NaOH and counted in 10 ml Instagel (Packard) in a Packard liquid scintillation counter. In set II ^{3}[H]thymidine incorporation was measured in logarithmically growing microplasmodia in liquid shake culture at 25 °C. The rate of incorporation in different cultures was standardized against either (*a*) protein content, estimated by the method of Lowry *et al.* (1951) or (*b*) the rate of protein synthesis estimated by the rate of incorporation of ^{14}[C] from a ^{14}[C]protein hydrolysate.

In method (*a*) 3 ml of a culture of microplasmodia was incubated together with 0·5 μCi/ml ^{3}[H]thymidine (Amersham, code TRK 120, specific activity 19 Ci/mmole).

After 15 min incubation, duplicate 1·0 ml samples were removed into 8·0 ml

ice-cold 5% TCA in acetone. The material was then treated as described for experimental set I, protein content and radioactivity being estimated after dissolving the final pellet in 1·0 ml 0·4 N-NaOH. In method (*b*) the same procedure as described in (*a*) above was used except that the culture was incubated with 5 μCi/ml ^{3}[H]thymidine (Amersham code TRK-120, specific activity 21 Ci/mmole) plus 0·125 μCi/ml ^{14}[C]protein hydrolysate (New England Nuclear) at specific activity 57 mCi/mmole. The final pellet was dissolved in 2·9 ml 0·4 N-NaOH and samples were counted in diluene (Packard).

(v) *Thymidine kinase assay*

The method adapted is based on Sachsenmaier & Ives (1965) and Hildebrandt & Sauer (1973). In experimental set I, synchronous plasmodia in early S phase were washed briefly in ice-cold water and then frozen in liquid nitrogen. The samples were either used immediately, or stored at −70 °C until the enzyme assay was performed. Samples were then thawed and sonicated on ice in 2 ml 50 mM Tris-HCl, pH 7·5, by three 1 sec pulses in an MSE sonicator (amplitude 5 μm with titan probe no. 43301, diameter $\frac{3}{8}$ inch). The sonicate was then centrifuged at 35000 rev/min in a Beckman L2-65B (rotor SW56) for 30 min. The thymidine kinase activity of the supernatant (enzyme extract) was then estimated by adding 20 μl extract to 100 μl incubation mixture containing: 10 mM ATP, 10 mM-D(−)-3-phosphoglyceric acid, 10 mM-$MgCl_2$, 2·5 mM EDTA-Na, 10 mM Mercaptoethanol, 20 mM Tris-HCl, pH 7·5, and 2·1 mM methyl-^{3}[H]thymidine, specific activity 1·2 Ci/mmole (TRK-120, Amersham). Incubation was at 28 °C and samples of the assay mixture were applied to Whatman DE-81 ion-exchange cellulose paper at appropriate time points. Descending chromatography with 0·1 N-NH_4OH in 4 N-HCOOH allowed separation of thymidine (R_F = 1) from thymidine monophosphate (R_F = 0·6).

Relative amounts of TMP formed could then be determined by cutting each chromatographic run into 15 fractions and counting each piece in the liquid scintillation counter in toluene PPO scintillation mixture.

In Experimental Set II, the enzyme extracts were prepared as described for set I and activity was measured using 100 μl incubation mixture and 100 μl extract. The incubation mixture differed from that of set I in that 80 mM Tris-HCl, pH 8·0, and 0·5 mM ^{14}[C]thymidine (1·4 μCi/μmole) were used. Incubation was at 28 °C and at appropriate times the reaction was stopped by placing the tubes in boiling water for 2 min followed by cooling in ice. After centrifugation to clear the mixture, duplicate 50 μl samples were applied to Whatman DE-81 paper squares (2 cm^2). The squares were washed twice for 20 min in 0·001 N ammonium formate to remove unreacted ^{14}[C]thymidine. This was followed by further washing for 2 min in water (twice), ethanol, and finally in acetone. The radioactivity (as bound ^{14}[C]TMP) was measured by placing the squares in 10 ml scintillant (Packard) and counting in a Packard liquid scintillation counter. This method was adapted from those described previously by Furlong (1963) and Taylor, Stafford & Jones (1972).

3. RESULTS

(i) *Induction and isolation of mutants*

In order to determine the optimal conditions for detecting BrdU-resistant mutants, small numbers of cells of an established mutant cell line, TU52, and of the wild type, TU4, were grown on BrdU substituted bacteria for three generations and exposed to varying doses of ultraviolet irradiation. Table 1 shows that 15 min gives a sufficient dose to differentiate clearly between mutant and wild type, while 75 min still allows near normal plating efficiency for this particular mutant strain.

Table 1. *Effect of UV dose on colony-forming capacity of TU52 and TU4 cells*

	Irradiation time (min)				
	0	15	25	40	75
TU52 colonies formed (mutant)	107	141	124	96	111
	119	111	119	71	83
TU4 colonies formed (wild type)	66	0	0	0	0
	66	0	0	0	0

Duplicate plates were inoculated with 100 cells per plate and irradiated daily for 8 days, as described in Methods.

Table 2. *Effects of cell density on the colony-forming ability of mutant cells under selective conditions*

TU4 wild-type cells spread per plate	TU52 mutant cells spread per plate	No. of colonies appearing
0	110	88
100	0	0
10^5	110	91
5×10^5	110	69
10^6	110	29

A small number (110) of mutant TU52 were grown with varying numbers of wild type TU4 on BrdU-substituted bacteria and irradiated daily for 35 min as described in Methods.

Information on the maximum number of cells that could be plated on one dish and still allow detection of mutant cells was obtained by plating a constant small number of mutant TU52 in the presence of large numbers of wild type TU4. Results displayed in Table 2 show that 10^5 to 5×10^5 cells is the upper limit for safe detection of mutant colonies. In three separate experiments where non-mutagenized cells were plated, mutants were found at frequencies of 10^{-5} to 10^{-6}. In two experiments where NG was used in attempts to improve mutant yield further, mutants were found at comparable frequencies. Thus there is no evidence that NG improves the yield of BrdU-resistant mutants in *Physarum*. (See Discussion.)

The mutants obtained could be classified as strong (5–8 mm), medium (2–5 mm) or weak (0·5–2 mm), according to colony diameter on days 5–7 when growing

on BrdU-substituted bacteria and being exposed to long wave UV light. Few weak mutants were analysed here since their analysis is particularly difficult due to phenotypic variation of the character studied in both mutant and wild type.

(ii) *Characterization of mutants*

(*a*) *Analysis of crosses between BrdU-resistant × wild-type strains*

In order to determine whether BrdU resistance in each of the 24 clones selected for further analysis was due to mutation of a single gene, each was crossed with a wild-type strain and the progeny classified for BrdU resistance and mating type. In cases where the mutants had been isolated in mt_h strains (set II) it was also possible to score for plasmodial markers (*fus*A, and in the cross *bur* 110 × LU657 also for *leu*-1$^-$) directly in plasmodia produced by the mt_h progeny clones. The results of the analysis of BrdU resistance and mating type are shown in Table 3.

These data suggest that in all cases BrdU resistance was due to mutation of a single gene since it segregates 1:1 from wild type. Further, there is no evidence of linkage between *bur* and *mt*.

(*b*) *Thymidine incorporation into plasmodia of mutant strains*

In order to test the mutants for a deficiency in thymidine incorporation, plasmodia of mutant strains were constructed and their ability to incorporate ^{3}[H]thymidine determined as described in section 2(iv).

Homozygous mutant plasmodia of the set I mutant strains were constructed by intercrossing BrdU resistant progeny amoebae of different mating types obtained during the analysis described in the previous section.

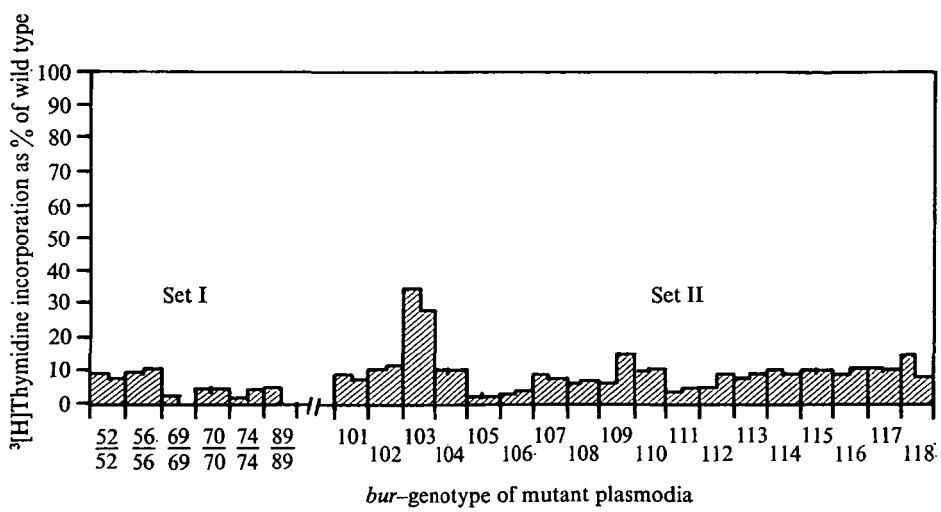

Fig. 1. Duplicate determinations of ^{3}[H]thymidine incorporation into plasmodia of *bur* genotype as indicated on abscissa. Plasmodia were constructed from bromodeoxyuridine resistant amoebae as described in the text. In set I, plasmodia were homozygous diploids in surface culture. Incorporation was normalized against plasmodial area. In set II, plasmodia were haploid and in shake culture. Incorporation was normalized against protein content (see Materials and Methods).

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Table 3. *Analysis of crosses between wild-type and BrdU-resistant strains*

Mutant × wild type	Mutant phenotype	*bur*+		*bur*	
		Set I			
		mt_1	mt_2	mt_1	mt_2
bur 52 × TU8	Strong	9	7	10	9
bur 56 × TU8	Weak	11	8	9	12
bur 69 × TU8	Strong	8	7	12	13
bur 70 × TU8	Strong	5	3	5	1
bur 74 × TU8	Strong	7	15	12	4
bur 89 × LU648	Strong	12	7	13	4
		Set II			
		mt_1	mt_h	mt_1	mt_h
bur 101 × LU853	Strong	5	7	5	6
bur 102 × LU853	Strong	4	8	5	6
bur 103 × LU853	Weak	15		14	
bur 104 × LU853		15		12	
bur 105 × LU853		5	3	8	3
bur 106 × LU853		4	10	5	3
bur 107 × LU853		5	4	2	5
bur 108 × LU853		13		16	
bur 109 × LU853		5	4	5	9
bur 110 × LU657		8	6	6	3
bur 111 × LU833	Strong	4	6	9	4
bur 112 × LU853		9	3	5	7
bur 113 × LU853		6	5	7	5
bur 114 × LU853		6	5	5	8
bur 115 × LU853		10	2	3	7
bur 116 × LU853		8	4	4	5
bur 117 × LU853		9		9	
bur 118 × LU853		4	6	7	7

Mutants and wild types were crossed as shown and progeny analysed for segregation of *bur*+ and *bur* phenotypes: In most cases, segregation of mating types (mt_1, mt_2 *or* mt_h) were also analysed.

(In all crosses the ratio of *bur* to *bur*+ progeny was not significantly different from 1:1 ($\chi^2 < 3{\cdot}8$; $P > 0{\cdot}05$).)

The set II mutant strains were of the genotype mt_h and mutant plasmodia could therefore be produced directly from the original clones of mutant amoebae.

It can be seen from the results in Fig. 1 that all mutants exhibited low thymidine incorporation into plasmodia when compared with wild-type controls.

It would thus appear that in the strains under investigation, the mutations causing BrdU resistance in the haploid amoebae also cause decreased rates of thymidine incorporation into plasmodia. It is therefore concluded that resistance in these mutants is the result of a defect in the ability of the cell to incorporate exogenous thymidine or its analogue, 5-bromodeoxyuridine.

It can be seen that one of the BrdU-resistant mutants classified as weak, *bur* 103, produced a plasmodium which was not as severely affected in its ability to incorporate exogenous thymidine as the remainder of the mutants.

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(*c*) *Complementation between* bur *mutants*

In order to test the functional relationships between the different *bur* mutants, heterozygous mutant plasmodia were constructed and their ability to incorporate exogenous thymidine was determined.

In set I, *bur* 52 was chosen as the common parental strain and mt_2 amoebae of the remaining five *bur* mutants (identified during the analysis described in (*a*) above) were crossed with it to produce heterozygous mutant plasmodia. In set II a mt_1, *fus*A2, *leu*-1$^-$, *bur* 114 strain, TU370 (identified during the analysis described in (*a*) above), was selected as the common parent and crossed with the remaining 17 *bur* mutants.

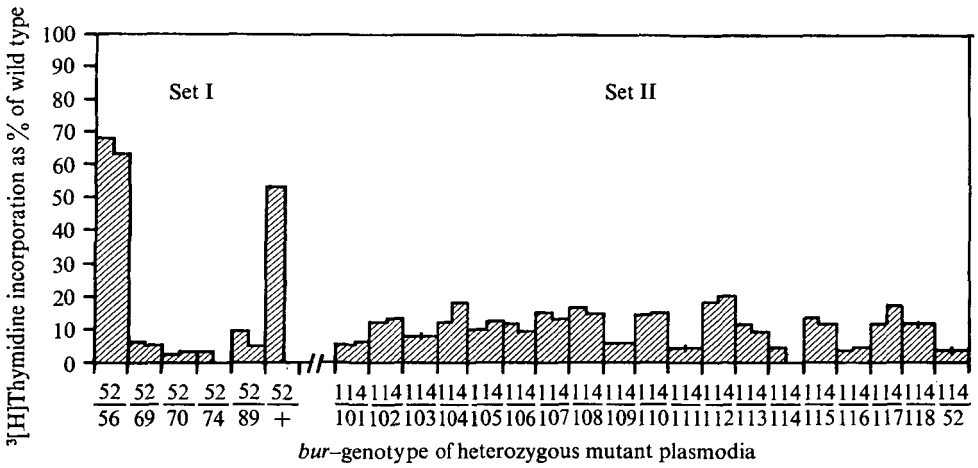

Fig. 2. Duplicate determinations of [^{3}H]thymidine incorporation into heterozygous mutant plasmodia. Plasmodia of the genotypes indicated on abscissa were constructed as described in the text. Set I data, obtained on surface cultures, were normalized against plasmodial area. Set II data obtained from shake-cultures, were normalized against rate of protein synthesis in each culture as measured by uptake of ^{14}[C]protein hydrolysate (see Materials and Methods).

The results (Fig. 2) show that in set I the plasmodium *bur* 52/*bur* 56 exhibited partial complementation, having a level of incorporation 60–70 % of that of the wild-type control. This figure is similar to that of a *bur* 52/*bur*$^+$ heterozygote (also included in Fig. 2). The remaining mutants had levels of incorporation similar to those of homozygous mutant plasmodia. It is therefore concluded that with the exception of *bur* 56 the mutants of set I are all defective in the same function and thus probably carry mutations of the same gene, designated *bur* A. The defective locus of *bur* 56 is accordingly designated *bur* B.

The results for set II (Fig. 2) suggest that mutants of this group all have the same functional defect, since all heterozygotes have a level of ^{3}[H]thymidine incorporation similar to that of the mutant *bur* 114 (the common parent), a level approximately 10% of that of the wild-type control. Further, the level of incorporation of the plasmodium *bur* 114/*bur* 52 would suggest that these are mutations of the same gene, previously designated *bur* A.

(*d*) *Recombination between* bur *mutants*

Further evidence that the mutations affecting *bur* 52 and *bur* 56 are mutations of different genes was obtained by analysing progeny of the plasmodium *bur* 52/*bur* 56 for wild-type recombinants. The results (Table 4) show that 4 out of a

Table 4. *Analysis of BrdU resistance amongst progeny of heterozygous mutant plasmodia*

Heterozygous mutant plasmodium	Progeny analysis BrdU	
	Resistant	Sensitive
bur 52/*bur* 56	16	4
bur 52/*bur* 74	20	0
bur 114/*bur* 101	45	0
bur 114/*bur* 103	45	0
bur 114/*bur* 115	45	0
bur 114/*bur* 52	32	0

Mutant clones were crossed in pairs as shown and progeny analysed for presence of wild-type recombinants.

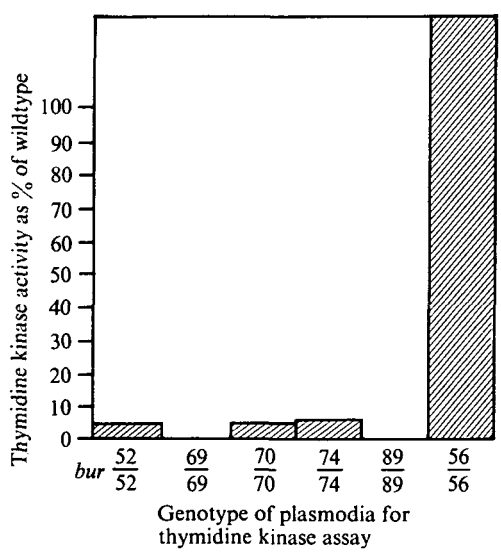

Fig. 3. Determinations of thymidine kinase activity in extracts of plasmodia homozygous diploid for bromodeoxyuridine resistance. Extracts were made and thymidine kinase activity determined as described in Methods. The activity in percentage of wild type, normalized to the protein content of the extract, is plotted against plasmodial genotype.

total of 20 clones analysed were BrdU-sensitive, thus confirming that *bur* A and *bur* B are two independent loci. As would be predicted on the basis of the complementation data above, no wild-type recombinants were detected amongst 20 progeny clones analysed of the plasmodium *bur* 52/*bur* 74. Similarly no wild-type recombinants were detected amongst the progeny of three randomly selected

heterozygous mutant plasmodia of set II, as would be predicted if all set II mutants are also defective at the *bur* A locus.

(*e*) In vitro *measurements of thymidine kinase activity of mutant plasmodia*

In vivo analysis showed that mutations causing BrdU resistance in the myxamoebae are paralleled by a deficiency of ^{3}[H]thymidine incorporation into plasmodia. To further characterize these mutations, the thymidine kinase activity of a *bur* B56/*bur* B56 plasmodium and several plasmodia of the genotype *bur* A/*bur* A was measured. The results (Fig. 3) showed that while mutations in *bur* A result in reduced thymidine kinase activity as compared to wild type, strains carrying a mutation at the *bur* B locus have no defect in thymidine kinase activity *in vitro*.

4. DISCUSSION

In the present investigation 24 BrdU-resistant mutants of *P. polycephalum* have been analysed. Twenty-three of these could be ascribed to a single locus, *bur* A, while one mutant appears to be at a second, probably unlinked locus, *bur* B. In the case of *bur* A mutants, BrdU-resistance in amoebae and lowered thymidine incorporation into plasmodia can be explained by a decrease in the ability of these cells to phosphorylate thymidine and thus also 5-bromodeoxyuridine, due to decreased thymidine kinase activity. An alternative mechanism must, however, be sought to explain BrdU resistance in strain *bur* B56. This mutant exhibited decreased thymidine incorporation into plasmodia but had a normal level of thymidine kinase activity. It may be significant that this mutant was one of the few selected for further analysis which belonged to the class of relatively weak mutants. In frog cells weak BrdU resistance was shown to correlate with a transport defect, while strong resistance was caused by a mutation resulting in low thymidine kinase activity (Freed & Mezger-Freed, 1973). Further experiments will be required to determine whether *bur* B56 does indeed have a transport defect.

Thymidine kinase has been shown to be a 'peak' enzyme in *P. polycephalum* with maximal activity correlated with the onset of mitosis (which in *P. polycephalum* is immediately followed by DNA synthesis) (Sachsenmaier, Fournier & Gürtler, 1967). Even though thymidine kinase is now commonly considered to function simply on a scavenge pathway and is probably not directly involved in the control of nuclear division or DNA synthesis, the study of mutants defective in this function may be of value in understanding the control of cyclic events associated with nuclear division. In HeLa cells thymidine kinase exists in three distinct molecular forms (Kit, Leung & Kaplan, 1973). Mutation of the cells to BrdU resistance results in the loss of one of these forms, that found in the cytosol fraction of cell extracts. The two remaining molecular forms are associated with a mitochondrial fraction and account for the residual enzyme activity in BrdU resistant cells. They suggest that one of the mitochondrial forms is a distinctive isoenzyme whilst the other may be a modified form of the cytosol enzyme.

In *P. polycephalum* Gröbner *et al.* (1975) have shown that thymidine kinase consists of three isoenzymes or enzyme variants, one of which reaches a peak at the time of mitosis.

They propose that this variant is formed at the time of induction of thymidine kinase (shortly before mitosis) and that it may be subsequently modified during the cell cycle to give rise to both the other variants.

Further experiments will determine which of the thymidine kinase variants of *P. polycephalum* are affected by mutation at the *bur* A locus.

One aim of the present study of BrdU resistant mutants was to provide information which might enable us to improve the efficiency of the BrdU suicide selection technique for the isolation of heat-sensitive mutants of *P. polycephalum* (Haugli & Dove, 1972). The power of this technique was reduced by its concomitant enrichment for BrdU-resistant mutants. Since the majority of BrdU resistant mutants obtained are severely deficient in thymidine kinase activity, they must depend on endogenous thymidylate synthesis. Thus, conditions which inhibit *de novo* thymidylate synthesis (via thymidylate synthetase) should prove lethal to thymidine kinase mutants. In most organisms it is possible to block the *de novo* synthesis of thymidylate using a variety of chemical inhibitors including the folate antagonists aminopterin or methotreaxate. These compounds inhibit dihydrofolate reductase thereby limiting the supply of tetrahydrofolate which is required in stoichiometric amounts during the thymidylate synthetase reaction. In preliminary experiments we have been unable, after aminopterin/methotrexate inhibition, to rescue wild-type cells by the addition of thymidine, methionine and adenosine. Thus, at present it would appear that it is not possible to improve the BrdU suicide selection technique for the isolation of heat-sensitive mutants by incorporating a regime which prevents enrichment for thymidine kinase mutants. One approach being attempted is the isolation of thymidylate synthetase negative (*thy*$^-$) mutants of *bur*$^+$ cells (Cooke, unpublished). Such cells would be dependent upon the continued action of thymidine kinase for growth.

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