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Complementation in balanced heterokaryons and heterozygous diploids of *Aspergillus nidulans*

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1. INTRODUCTION

The life-cycle of the mould *Aspergillus nidulans* contains three contrasting levels of nuclear-cytoplasmic organization, haploid organisms, balanced heterokaryons and heterozygous diploids (Pontecorvo *et al.*, 1953; Roper, 1952), which present a novel system for the analysis of cellular organization and the study of mechanisms of gene action (Pontecorvo, 1952; Roper, 1958). Relatively little has been done with *Aspergillus* in the investigation of gene action by biochemical techniques (Shepherd, 1956; Roberts, 1963*b*; Cove & Pateman, 1963), and the unique situation with regard to levels of organization remains unexplored.

Pontecorvo (1952) has suggested that genes combined at different levels of organization may exhibit different phenotypes. A number of examples of such differences have been described recently in both the *intra-* and *inter*-genic complementation of mutants combined in the *trans* arrangement in either heterokaryons or in diploids. One example of an *intra*-genic difference are certain recessive mutants at the *pan-2* locus in *Neurospora* which fail to complement in heterokaryons although they complement in disomic 'pseudowild type' strains (Case & Giles, 1960). Another example has been detected in the complementation of recessive sorbitol mutants in heterokaryons or diploids of *Aspergillus*, but is was not clear if this was an *intra-* or an *inter*-genic difference (Roberts, 1963*a*). Two intriguing examples of a failure of *inter-genic* complementation between loosely linked recessive suppressor mutants in dikaryons of *Coprinus* have been described (Lewis, 1961; Morgan, 1961), but until stable diploid strains of this mould have been isolated the phenotype resulting when the mutants are in the same nucleus remains unknown.

Previous work (Roberts, 1963*a*) showed that wild-type *A. nidulans* utilizes sorbitol as a carbon source for growth and will grow on defined media in which sorbitol is the sole carbon source. Two types of sorbitol mutant were isolated after ultraviolet irradiation; total mutants (*sb3*, *sb5*) which failed to grow at all on the sugar, and leaky mutants (*sb4*, *sb6–sb9*) which yielded characteristic poor growth. The mutants were all recessive, and during functional analysis the unusual result was obtained that although certain pairs of mutants complemented in heterozygous diploids, none complemented in balanced heterokaryons. Complementation of the

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mutants in diploids is described in Fig. 1 in the form of a simple complementation map. The mutant *sb8* was non-complementary to all the other mutants. The remaining mutants were complementary in all combinations except *sb3*+*sb5*, *sb4*+*sb7* and *sb6*+*sb9*, and these three pairs of mutants therefore defined three complementation groups between which complementation occurred but within which it did not occur. Combinations of total+total mutant or leaky+leaky mutant yielded, if non-complementary, the expected total or leaky phenotypes. Combinations of total+leaky mutant yielded the less severe leaky phenotype when non-complementary.

sb8

sb3,5 *sb4,7* *sb6,9*

Fig. 1. Complementation of sorbitol mutants in heterozygous diploids of *Aspergillus nidulans*.

Further investigation of the sorbitol mutants of *Aspergillus nidulans* is reported in this paper. A quantitative technique permits demonstration of the difference between complementary (wild-type) and non-complementary (leaky) growth, and this difference is correlated with a difference in rates of sorbitol oxidation by intact mycelium. The mutants are shown to be closely linked, but the critical question of *inter*- or *intra*-genic complementation remains undecided, although the evidence available is slightly in favour of *inter*-genic complementation.

2. METHODS

Organisms. The sorbitol mutants were isolated from the Glasgow strain *bi1; w3* of *Aspergillus nidulans* following ultraviolet irradiation (Roberts, 1963*a*). They were recombined through the sexual cycle (Pontecorvo *et al.*, 1953) with two sets of markers derived from strains in the Glasgow collection. The pedigrees of the strains used in the present investigation were as follows: Type A strains, *gal 5 bi1; w3* (U.V. mutant of *bi1; w3*) × *paba1 y ad20* → *gal5 paba1 y ad20* × *su1ad20 ribo1 pro1 ad20 bi1; pyro4* → *ribo1 pro1 y ad20* × *bi1; w3; sb* → *ribo1 pro1 bi1; w3; sb.* Type B strains, *bi1; w3; sb* × *ad14 paba1 y; sb.*

Symbols of mutant alleles: requirements, *bi1* = biotin; *paba1* = *p*-aminobenzoic acid; *ad20*, *ad14* = adenine; *pro1* = proline; *pyro4* = pyridoxine; *ribo1* = riboflavine; *su1ad20* = suppressor of *ad20*. Sugar marker, *gal5*, no growth on galactose. Conidial colours, *w3* = white; *y* = yellow; the wild-type is green.

Standard techniques were followed in the synthesis of balanced heterokaryons (Pontecorvo *et al.*, 1953) and in the isolation of heterozygous diploid strains (Roper, 1952). The diploid strains were not sub-cultured more than once after isolation. Strains were maintained on slopes of supplemented malt extract agar.

Media. Malt extract agar (Roberts, 1963*b*) was supplemented with biotin, adenine, *p*-aminobenzoic acid, riboflavine, and proline at the concentrations normally added to minimal medium (Pontecorvo *et al.*, 1953). Basal medium and basal agar (basal medium solidified with 2·5% British Drug Houses' agar) were the standard minimal media for *Aspergillus nidulans* (Pontecorvo *et al.*, 1953) prepared

without the carbon source and including a trace salts solution (Roberts, 1963*b*). Carbohydrates were dissolved in distilled water and sterillized by autoclaving at 10 lb. pressure for 10 min. They were added to cool molten basal agar or to basal medium at the rates indicated. Biotin was supplied in excess (0·5 mg./l.) to liquid media which also contained the wetting agent Tween 80 (0·1 ml./l.) to bring conidia into suspension. Haploid or diploid strains were grown in submerged culture at 30° with vigorous aeration and harvested as homogenous suspensions of small mycelial pellets (Roberts, 1963*b*).

Estimations. Manometric assays of O_2 uptake were done by conventional methods (Umbreit, Burris & Stauffer, 1949). The manometer flasks contained a suspension of mycelial pellets in 40 mM potassium phosphate buffer at pH 6·5 (2·0 ml.; equivalent to 2–5 mg. dry wt. mycelium) in the main compartment, 0·5 ml. of 10 mM substrate solution (or 0·5 ml. water for the determination of endogenous rates) in the side arm and 0·2 ml. of 20% KOH in the centre well. The temperature was 30°. There was a linear relationship between oxygen uptake and dry wt. of mycelium up to 7·5 mg. dry wt. mycelium/flask.

Chemicals. Analytical grade chemicals were used when available. D-Sorbitol (laboratory reagent) and glucose-6-phosphate were from British Drug Houses, Poole, Dorset. Sorbitol-6-phosphate was prepared by borohydride reduction of glucose-6-phosphate (Wollf & Kaplan, 1956).

3. RESULTS

(i) *Measurement of growth on solid media*

The classification of pairs of sorbitol mutants as complementary or non-complementary when combined in balanced heterokaryons or in heterozygous diploids, is a qualitative description of whether the growth of the mycelium on basal agar and sorbitol is more like that of the wild-type or the mutant. It was desirable to have a quantitative measure of growth, and as the yield of haploid or diploid mycelium in liquid culture is readily determined attempts were made to grow heterokaryons in this way.

Balanced heterokaryons were synthesized between strains with either white or yellow conidia and with complementary nutritional requirements (*ribo1 pro1 bi1; w3; sb3* and *ad14 paba1 y; sb4*). They were maintained on basal agar plus glucose. Heterokaryotic mycelium was transferred to liquid basal medium plus glucose either by inoculation with blocks of agar containing young, actively growing hyphae or with sheets of heterokaryotic mycelium grown between two semipermeable membranes on the surface of solid medium (see below). In both cases large quantities of mycelium growing as unorganized masses of cells were obtained after 4 days incubation at 30° in shake culture. However, this mycelium was mainly composed of diploid cells, since small fragments transferred to basal agar plus glucose yielded only colonies with green spores. (White conidia or yellow conidia are determined by recessive mutations of two unlinked genes and thus a heterozygous diploid w^+y/wy^+ has the wild phenotype with respect to conidial colour.) In the present case it was

evident that diploid mycelium had a considerable advantage over heterokaryotic mycelium in liquid culture, perhaps due to leaching of metabolites from the heterokaryon. Rare diploid nuclei either present in the original mycelium or arising early in growth in liquid culture gave rise to diploid strains which rapidly outgrew the heterokaryon.

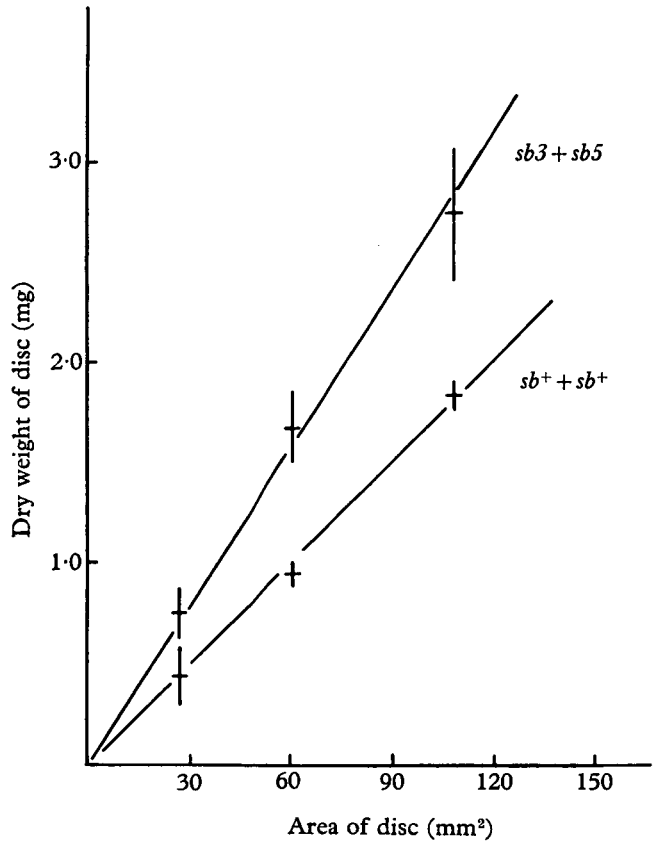

Fig. 2. Relationship of disc area and dry weight of mycelium. The balanced heterokaryons (*ad14 paba1 y; sb3+bi1; w3; sb5* and *ad14 paba1 y+bi1; w3*) were grown on basal agar + 1% glucose for 7 days at 37°. The mean dry weights of sets of five discs of different diameters cut from each heterokaryon were determined as described in the text. The vertical lines show the standard deviation of means.

It became necessary, therefore, to devise a technique to measure the growth of organisms on solid media, and the characteristic leaky growth of the sorbitol mutants suggested the following method. A standard volume of agar was poured in large petri dishes (15 cm. diameter) to form thick agar plates. Colonies were incubated until they ceased growing when discs were cut from the mycelium with a cork-borer and the agar dissolved by autoclaving the discs individually in 20 ml. of distilled water for 10 min. at 10 lb. pressure. The discs of mycelium remained intact and were rinsed with hot water, dried at 105° for 18 hr. and weighed on a micro-balance.

Generally four colonies, each of a different strain, were grown in each dish and eight discs cut from each colony. Dishes were set up in triplicate. The validity of the technique was demonstrated in two ways. First, when different-sized discs were cut from the same colony it was found that the mass was proportional to the area of the disc (Fig. 2). Second, replicates were in close agreement (the standard deviation was about 20% of the mean) and two sets of heterokaryons measured on different occasions yielded results which did not differ significantly.

(ii) *Growth of haploid strains with glucose or sorbitol as carbon source*

The technique just described was used to measure the growth of the original strain *bi1; w3* and the sorbitol mutants on basal agar supplemented with biotin

Table 1. *Growth of* Aspergillus nidulans *strain* bi1; w3 *and the sorbitol mutants on solid media*

	Carbon source for growth			
	Glucose		Sorbitol	
Strain	Phenotype	Dry weight (0·01 mg.)	Phenotype	Dry weight (0·01 mg.)
bi1; w3	W	146 ± 34	W	67 ± 18
bi1; w3; sb3	W	150 ± 27	T	0
bi1; w3; sb4	W	137 ± 37	L	22 ± 04
bi1; w3; sb5	W	138 ± 27	T	0
bi1; w3; sb6	W	164 ± 41	L	25 ± 04
bi1; w3; sb7	W	171 ± 32	L	31 ± 12
bi1; w3; sb8	W	175 ± 27	L	42 ± 08
bi1; w3; sb9	W	178 ± 26	L	45 ± 12

Phenotypes: W = Wild-type. T = Total mutant. L = Leaky mutant.
Strains were grown at 37° on basal agar + biotin + 1% (w/v) glucose (5 days) or 1% (w/v) sorbitol (7 days). The dry weights of standard discs of mycelium were measured by the method described in the text. Each figure is the mean for 24 discs.

and either glucose or sorbitol (Table 1). All the strains grew equally well on glucose, whereas the leaky sorbitol mutants yielded only 30–60% of the growth of the wild-type on sorbitol.

(iii) *Growth of balanced heterokaryons and heterozygous diploids on sorbitol*

The growth of two sets of balanced heterokaryons and the corresponding diploids on basal agar + sorbitol was measured (Fig. 3). The two total mutants *sb3* and *sb5* failed to grow when combined either in heterokaryons or in diploids. They were thus non-complementary in both systems and attention is directed in Fig. 3 to pairs of mutants other than *sb3* + *sb3* and *sb3* + *sb5*. In the heterokaryons the growth of

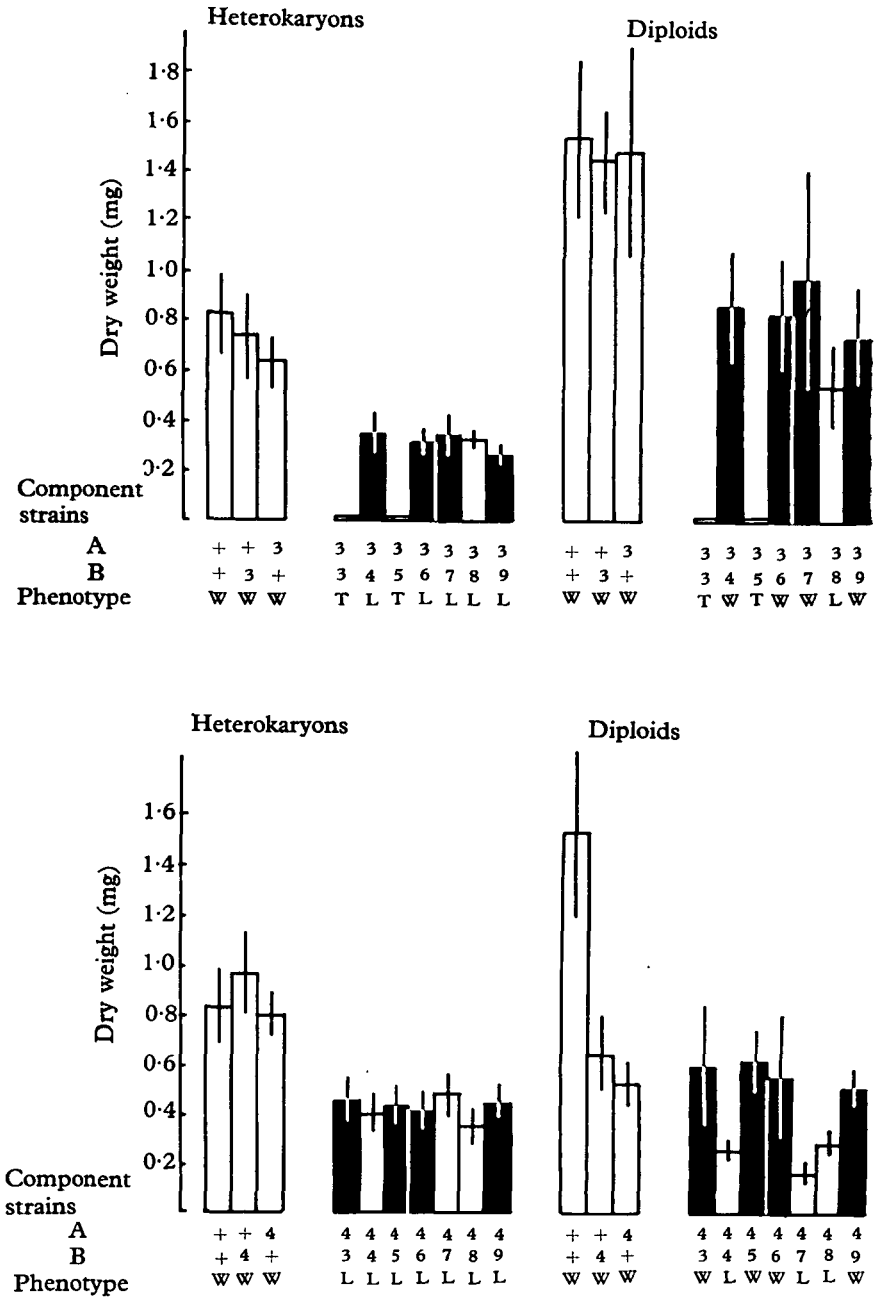

Fig. 3. Growth of balanced heterokaryons and heterozygous diploids on sorbitol. The heterokaryons were grown on unsupplemented basal agar + 1% sorbitol for 7 days at 37° and the diploids for 5 days. The histograms represent the mean dry weights of discs of mycelium cut from three different colonies of each heterokaryon or diploid and are blocked in for pairs of mutants which complement in heterozygous diploids. The vertical lines show the standard deviations. The phenotypes of the different combinations are indicated as in Table 1. Component strain A = *ribo1 pro1 bi1; w3; sb;* B = *ad14 paba1 y;sb*

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all the combinations of mutants was the same and corresponded to their leaky phenotypes. However, in the diploids the growth of the same combinations of mutants clearly separated into two groups which corresponded to the leaky mutant or wild phenotypes, and reveal the non-complementary or complementary pair of mutants. This was particularly clear in the *Asb4* series of diploids where there were three non-complementary and four complementary combinations. Complementary pairs of mutants yielded about 50% of the growth of wild-type controls in the *Asb3* series of diploids. In the *Asb4* series, however, the growth of all the complementing mutants was only about 30% of the +/+ control. The heterozygous controls +/*sb4* and *sb4*/+ also yielded the same amount of mycelium suggesting that the *sb4* gene can diminish growth, although this effect was not apparent in the diploid *Asb3*/*Bsb4*.

The growth of the same two series of heterokaryons and diploids on basal agar plus glucose was also measured. The controls produced about three times the weight of mycelium compared with growth on sorbitol and the combinations of mutants yields within the range of 80–120% of the controls.

(iv) *The adaptive oxidation of sorbitol by intact mycelium*

The difference in complementary growth of the mutants combined in balanced heterokaryons or in heterozygous diploids may result from a difference in the amount of an enzyme formed in the two systems, and to test this the rates of oxidation of sorbitol by intact mycelium were determined.

Table 2. *The adaptive oxidation of sorbitol by* Aspergillus nidulans, *strain* bi1; w3

Carbon source for growth	Rates of O_2 uptake (μl./hr./mg. dry weight mycelium)		
	Endogenous	Glucose*	Sorbitol*
Glucose (1%)	10·2	25·1	0
Sorbitol (1%)	15·4	23·8	28·6

* Values less the endogenous rates.

Pellets of mycelium were grown for 15 hr. at 30° in basal medium + biotin + carbon sources indicated. Rates of oxidation were determined manometrically.

The ability of *Aspergillus nidulans* to oxidize sorbitol was an inducible property which depended upon the presence of sorbitol in the growth medium (Tables 2, 4). Adaptation to sorbitol was rapid and maximum rates of oxidation were obtained after 4 hr. induction (Table 3). Glucose repressed induction of the enzyme system since mycelium grown on glucose + sorbitol failed to oxidize sorbitol. However, induction was not repressed by glycerol and it was most convenient to induce strains by growth on glycerol plus sorbitol. The sorbitol mutants were all tested in this way (Table 4). It was found that, as with the original strain, none of the sorbitol mutants oxidized sorbitol at an appreciable rate when grown in the absence of the

sugar. When grown under inducing conditions the leaky mutants failed to oxidize sorbitol, but the total mutants oxidized it at 50–70% the rate of the wild-type.

Table 3. *Induction of the sorbitol oxidase system in* Aspergillus nidulans, *strain* bi1; w3

Time (hr.)	Dry weight of mycelium (mg./ml.)	Rates of O_2 uptake (µl./hr./mg. dry weight mycelium)	
		Endogenous	Sorbitol*
0	0·55	15·9	0
2	0·48	13·7	2·5
4	0·50	20·8	23·9
6	0·65	22·2	23·0
9	0·93	25·0	23·9
12	1·61	21·2	21·0

* Values less the endogenous rates.

Pellets of mycelium were grown for 15 hr. in basal medium + biotin + 1% glucose. They were washed aseptically by centrifugation, resuspended in fresh basal medium + biotin + 1% sorbitol and incubated at 30°. Samples were taken at the times indicated, the rates of sorbitol oxidation assayed manometrically and the dry weight of mycelium determined.

Table 4. *The oxidation of sorbitol by sorbitol mutants of* Aspergillus nidulans

Strain	Growth on sorbitol	Rates of O_2 uptake (µl./hr./mg. dry weight of mycelium)			
		Non-induced		Induced	
		Endogenous	Sorbitol*	Endogenous	Sorbitol*
bi1; w3	W	20·4	1·2	18·3	16·4
bi1; w3; sb3	T	17·9	0·9	18·1	8·0
bi1; w3; sb4	L	21·0	1·0	24·9	1·1
bi1; w3; sb5	T	19·0	1·6	23·8	12·0
bi1; w3; sb6	L	21·2	2·5	19·6	0
bi1; w3; sb7	L	17·9	1·7	23·5	3·7
bi1; w3; sb8	L	21·2	0	20·8	1·8
bi1; w3; sb9	L	19·4	0·8	20·6	0

* Values less the endogenous rates.

Pellets of mycelium were grown for 18 hr. at 30° in basal medium + biotin + 2% glycerol (non-induced mycelium) or 2% glycerol + 1% sorbitol (induced mycelium). The phenotypes on sorbitol are described as in Table 1.

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This result suggested that there were at least two enzymes concerned in the utilization of sorbitol, and initial oxidative enzyme (A) and a later enzyme (B). Total mutants form enzyme A, and thus oxidize sorbitol, but lack enzyme B. Leaky mutants are defective in enzyme A and leaky growth could result either from low levels of enzyme A activity or the operation of an alternate metabolic pathway. Failure of the total mutants to grow on sorbitol may then result either from the loss of both pathways or the inhibition of growth by the accumulation of the product of the initial oxidative enzyme (A) reaction.

(v) *The oxidation of sorbitol by balanced heterokaryons and heterozygous diploids*

The diploid strains were induced by growth in basal medium plus 2% glycerol and 1% sorbitol for 18 hours at 30°. The standard method was employed in which cultures were started from a heavy inoculum of conidia and yielded a fine homogenous suspension of small pellets of enmeshed mycelium. Such a method could not be used for the heterokaryons because they break down into their component strains when conidia, which are uninucleate, are formed (Pontecorvo *et al.*, 1953). Since heterokaryons were rapidly overgrown by diploid strains in liquid media it was again necessary to devise a method based on growth on solid media. The heterokaryons were grown on the surface of basal agar plus 1% glucose sandwiched between two semi-permeable membranes ('Visking' dialysis tubing, Gallenkamp & Co. Ltd., London). A small block of agar containing actively growing heterokaryotic mycelium was used as an inoculum and after 5 days' incubation at 37° had produced a circular sheet of mycelium occupying some 75% of the area of the plate. The membranes and mycelium were then transferred to fresh basal agar plus 1% sorbitol and incubated for a further 18 hr. at 37°. The mycelium did not spore except in a central area of about 3 cm. diameter where older hyphae had penetrated the upper membrane and formed conidial heads. This central area was cut out and discarded, the outer ring of mycelium cut radially into sixteen pieces and the membranes floated off under water. The mycelium was washed thoroughly in distilled water and finally suspended in 40 mM phosphate buffer (pH 6·5). A fragment was taken from each of the pieces of mycelium and grown on basal agar plus glucose to test for the possible occurrence of diploid sectors. Diploids were detected by the appearance of green-spored colonies and in the one case that they occurred the assay was repeated with anew heterokaryon.

In the manometric assay for O_2 uptake four pieces of mycelium were tested in each of four Warburg flasks containing 2·5 ml. of phosphate buffer in the main compartment, 0·2 ml. of 20% KOH in the centre well and 0·5 ml. of 500 mM sorbitol in the side arm. The bath temperature was 30°. Oxygen uptake due to respiration of endogenous substrates was measured for 2 hr. at which time the sorbitol was added and O_2 uptake followed for a further 2 hr. Finally, the mycelium in each flask was collected, washed thoroughly, and its dry weight determined. Each heterokaryon was tested in duplicate. No oxygen uptake could be detected unless a high concentration of substrate was added; this may be required to achieve penetration of

Table 5. *The oxidation of sorbitol by balanced heterokaryons and heterozygous diploids of* Aspergillus nidulans

		Genetic composition		Heterokaryons			Heterozygous diploids		
				Phenotype	Rates of O_2 uptake		Phenotype	Rates of O_2 uptake	
		A	B		Endogenous	Sorbitol*		Endogenous	Sorbitol*
Induced mycelium	Wild-type controls	*sb*+	*sb*+	W	9·4	4·9	W	20·6	15·6
					8·9	4·4			
		sb+	*sb4*	W	10·7	8·9	W	16·8	18·2
					10·1	9·5			
		sb4	*sb*+	W	9·0	4·0	W	20·6	17·6
					6·4	4·1			
	Non-complementary mutants	*sb4*	*sb4*	L	11·3	1·9	L	15·3	1·7
					12·2	1·3			
		sb4	*sb7*	L	12·4	2·4	L	18·1	0·8
					10·9	3·1			
		sb4	*sb8*	L	12·8	2·2	L	19·9	1·9
					11·2	1·9			
	Complementary mutants	*sb4*	*sb3*	L	10·3	1·4	W	13·8	13·8
					12·9	2·7			
		sb4	*sb5*	L	9·5	1·3	W	17·5	14·4
					10·1	1·3			
		sb4	*sb6*	L	11·7	1·9	W	18·6	9·8
					11·5	1·7			
		sb4	*sb9*	L	13·4	1·2	W	20·4	14·0
					13·6	1·2			
Non-induced mycelium		*sb*+	*sb*+	—	9·5	2·2	—	17·5	0·5
					8·4	2·4			

* Values less endogenous rates.

Rates of O_2 uptake = μl./hr./mg. dry weight mycelium.
Symbols for phenotypes as in Table 1. Component strains: A = *ribo1 pro1 bi1*; *w3*; *sb*; B = *ad14 paba1 y*; *sb*.
Mycelium was treated by procedures described in the text. The two sets of figures for the heterokaryons refer to duplicate experiments.

the sheet of hyphae. Each Petri dish yielded 10–15 mg. dry weight of mycelium, which was equivalent to about 5 mg. of protein in a crude cell-free extract. This general technique may be suitable for study of enzymes in heterokaryons of *Aspergillus*.

Rates of sorbitol oxidation were low in the heterokaryons compared with the diploids (Table 5) or haploids (Table 4). This was probably a result of the difference in growth form of the mycelium and the conditions of culture. In the balanced heterokaryons oxygen uptake by induced wild-type controls increased 2–4 times compared with non-induced controls. None of the pairs of mutants showed significant increase in oxygen uptake after induction and they all apparently failed to oxidize the substrate. On the other hand, in the heterozygous diploids complementary pairs of mutants oxidized sorbitol at about 75% of the rate of the wild-type controls but the non-complementary mutants failed to oxidize the substrate. The phenotypic differences observed between the mutants combined in heterokaryons or in diploids with regard to their growth upon sorbitol were therefore correlated with differences in their ability to oxidize the sugar.

The next stage in the analysis of the mutants would be identification of the precise metabolic lesions. However, all attempts to detect the individual enzymes of sorbitol metabolism were unsuccessful. Cell-free extracts of the wild-type organism grown upon sorbitol were prepared by the method of Hughes (1951), by aqueous extraction of acetone powders or by grinding in the cold with glass powder. They were tested for (*a*) the ability to phosphorylate sorbitol in the presence of ATP using a coupled enzyme assay to detect ADP production (Kornberg & Pricer, 1951), (*b*) direct oxidation of sorbitol by molecular oxygen using a manometric technique, and (*c*) the dehydrogenation of either sorbitol or sorbitol-6-phosphate in the presence of di- or tri-phosphopyridine nucleotides (DPN or TPN). The extracts contained negligible activity of DPNH or TPNH oxidase. No activity could be detected in any of the assays although the extracts did contain an active hexokinase and a very active glucose-6-phosphate dehydrogenase (TPN linked). It is probable that fructose is an intermediate in sorbitol utilization by *A. nidulans* since mutants unable to utilize fructose also fail to utilize sorbitol (Roberts, 1963*a*). However, no accumulation of fructose by the total mutants, which oxidize sorbitol, could be detected when they were incubated with sorbitol in shake culture.

(vi) *Crosses between the sorbitol mutants*

Crosses between mutants representing each of the complementation groups were analysed and wild-type (sb^+) organism isolated by plating ascospores from single hybrid perithecia in a selective medium containing sorbitol as sole carbon source (Table 6). Heavy background growth occurred, but wild-type colonies were distinguishable if not more than 10^5 ascospores were tested per plate.

No wild-type organisms were isolated in crosses homozygous for the sorbitol mutants and therefore the reversion rates of the mutants are sufficiently low to be ignored in the present experiments. In crosses between the mutants, wild-type organisms were only isolated at low frequencies and it was evident that the mutants

were closely linked. No wild-types were isolated from crosses including *sb8*, but they were isolated from crosses between complementary mutants and could have originated from aneuploid or diploid ascospores (Pritchard & Pontecorvo, 1953). Unstable aneuploids were distinguished by replating the sb^+ strains twice on a non-selective (supplemented malt extract agar) and then re-testing growth on sorbitol. In a number of cases (for example *sb3* × *sb4*) sectoring colonies typical of aneuploids were observed and the two types of sorbitol mutant could be isolated from different sectors (Käfer, 1960). In other cases the purified strains failed to

Table 6. *Crosses between sorbitol mutants in* Aspergillus nidulans

Cross: $\frac{ribo1 \quad + \quad pro1 \quad + \quad + \quad bi1 \quad w3 \quad sbx \quad +}{+ \quad ad14 \quad + \quad paba1 \quad y \quad + \quad + \quad + \quad sby}$

Cross *sbx* × *sby*	Ascospores tested: Total × 10^6	Ascospores tested: Mean No./plate × 10^3	sb^+ organisms isolated: Total	Aneuploids	Diploids	Haploids	sb^+ haploids/10^6 ascospores	
3 × 3	—	—	—	—	—	—	—	
4 × 4*	1·98	95	0	—	—	—	0	
6 × 6*	0·46	26	0	—	—	—	0	
8 × 8*	2·32	115	0	—	—	—	0	
3 × 4	0·24	21	18	6	0	12	50	
	0·28	25	17	2	0	15	54	74
	0·46	33	50	5	0	45	98	
3 × 6	0·06	14	11	2	1	8	133	
	0·13	44	43	6	0	37	284	180
	0·27	37	42	5	0	37	137	
3 × 8*	0·96	48	0	—	—	—	0	
4 × 6	1·27	127	47	6	0	41	32	
	0·66	66	40	4	1	35	53	44
	0·74	74	54	12	0	42	57	
8 × 4*	3·10	155	0	—	—	—	0	
8 × 6*	0·70	35	0	—	—	—	0	

* Data for three hybrid perithecia have been combined.

In each cross the ascospores from three hybrid perithecia were separately suspended in 12 ml. of saline. Samples of 1·0 ml. of the suspensions were mixed with cool (50°) molten basal agar containing the nutrients required by the parental strains and 1% sorbitol as carbon source. The mixture was poured as a middle layer sandwiched between two layers of the same medium, and the plates incubated for 4 days when sb^+ organisms had grown through to the surface of the medium and were sporulating. The numbers of viable ascopores tested were determined by plating suitable dilutions of the original ascospore suspensions in the same medium but with 1% glucose replacing sorbitol as the carbon source.

grow on sorbitol and were presumed to have been unstable aneuploids which had broken down during transfer on non-selective medium. The diameters of the conidia of all the remaining wild-types were measured and a few diploid strains were detected (Pontecorvo, Tarr, Gloor & Forbes, 1954). The remaining sb^+ strains could either have been authentic haploid recombinants, stable aneuploids disomic for the sorbitol chromosome, or possibly strains with duplications heterozygous for the sorbitol genes. These alternatives cannot be distinguished without closely linked markers. However, the frequency at which the wild-types were isolated from different

perithecia in each cross was reasonably constant and was characteristic of the cross; this is consistent with the interpretation that the *sb*+ organisms were recombinants. In this case *sb8*, which apparently failed to recombine with either *sb3*, *sb4* or *sb6*, although these recombined with one another, has the character of a deletion spanning three functionally distinct but closely linked genes.

4. DISCUSSION

The phenotypic situation is summarized in Fig. 4 in which data for wild-type controls and for complementary or non-complementary groups of mutants have been combined. If the mutants had complemented in the heterokaryons and attained the same proportion of the values of the controls that they do in the diploids, then the expected levels of growth and of sorbitol oxidation in the heterokaryons would be those indicated in Fig. 4. In both cases there was a significant difference between expected and observed values, and it was clear that mutants which complemented in heterozygous diploids failed to complement in balanced heterokaryons.

The heterokaryons grew normally on basal medium containing glucose and continued to grow vigorously when returned to this medium from sorbitol test media (Roberts, 1963*a*). Failure to complement was obviously not due to failure in heterokaryon formation (Grindle, 1963). The mutants did not complement when the test media were supplemented with nutrients required by the component strains, that is when an opportunity was provided for a shift in nuclear ratios, and also when combined with different sets of nutritional markers. These results suggested that failure to complement was not the result of an unfavourable nuclear ratio or of a nutritional interaction in the heterokaryons.

The genetic situation has been complicated by the finding that the original strain of *A. nidulans* (*bi1; w3*) from which the sorbitol mutants were isolated contains at least two translocations, one of which involves chromosomes VI and VII (Käfer, 1963). Both translocations have been found in one of the mutants *bi1; w3; sb3* (Käfer, personal communication) and they are most likely to be in the other mutants also. Preliminary mitotic analysis had located three of the mutants in chromosome VI (Roberts, 1963*a*), but this was done with Tester strains not marked in all of the chromosomes (Forbes, 1959) and the mutants could have been in either chromosome VI or VII.

The discovery of the chromosome VI-VII translocation raises the question whether the difference in complementation in the heterokaryons and diploids may be in some way associated with heterozygosity for the translocation. However, this seems unlikely since the complementation pattern was strictly a property of the sorbitol mutants, and was precisely the same in two sets of diploids synthesized between strains in which the mutants had been recombined through the sexual cycle with different sets of nutritional markers (i.e. *bi1; w3; sb+ad14 paba1 y; sb;* and *ad14 paba1 y; sb+ribo1 pro1 bi1; w3; sb*). The translocation was presumably segregating in these crosses but evidently did not affect the complementation patterns of the mutants. One of the strains used in the crosses (*ad14 paba1 y*) probably has not any translocations (Käfer, personal communication).

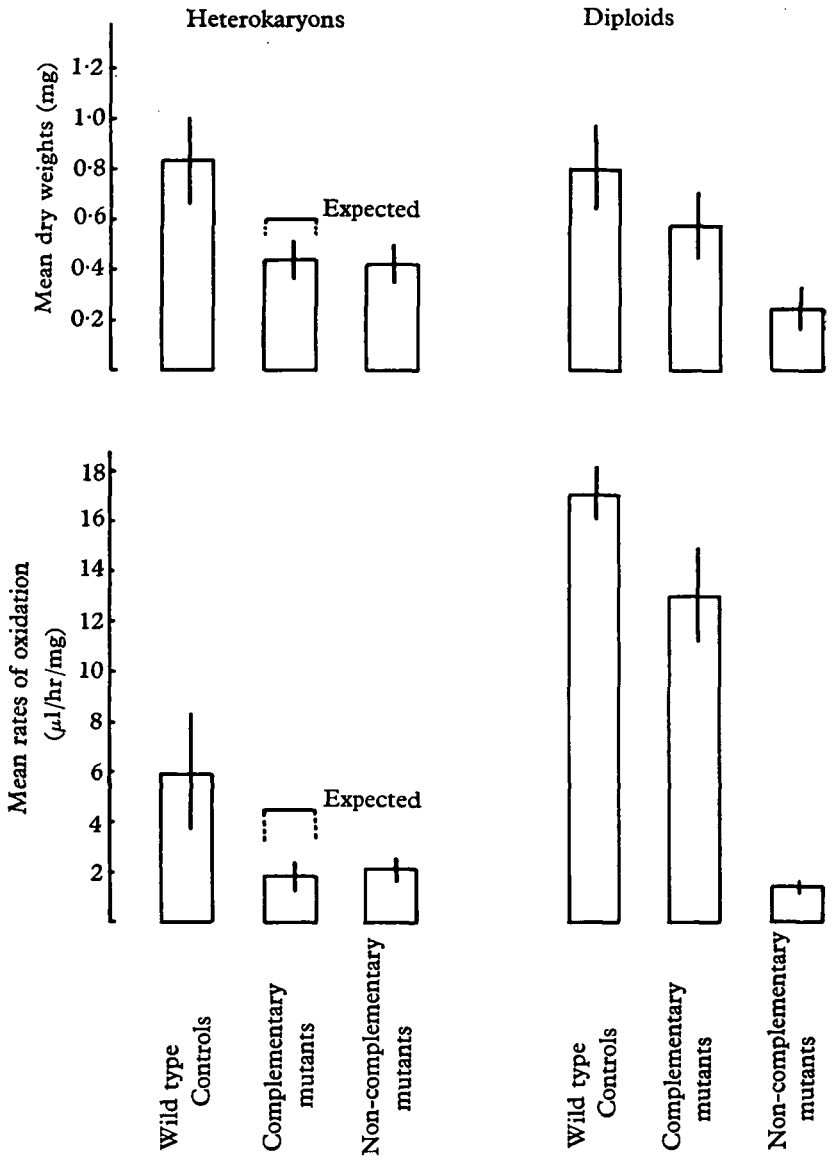

Fig. 4. Complementation of sorbitol mutants in balanced heterokaryons and heterozygous diploids of *Aspergillus nidulans*. The dry weights of mycelium (Fig. 3) and rates of sorbitol oxidation (Table 5) for wild-type controls, complementary and non-complementary mutants in the *Asb4* series have been pooled and the mean values shown in the form of histograms for each group in heterokaryons and in diploids. The vertical lines show the standard deviation of means. The 'expected' values are those predicted if the mutants did complement in heterokaryons and attained the same percentage of the controls that they do in diploids.

The possibility that mitotic crossing-over in the diploids, yielding a *cis* arrangement of the mutants and one wild-type chromosome, was the origin of the complementary growth of the diploids can be ruled out since the sorbitol mutants are

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closely linked and mitotic crossing over is a rare event in *A. nidulans* (Pontecorvo, Tarr-Gloor & Forbes, 1954). Also the complementing wild-type colonies were clearly not developing from sectors when the diploids were tested for growth on sorbitol. The diploids have not been tested for the recovery of the component sorbitol mutants on haploidization, but both mutants have been recovered from unstable aneuploids selected by their wild-type growth on sorbitol in the recombination experiments.

Failure to grow heterokaryons in submerged culture compelled the measurement of rates of sorbitol oxidation in heterokaryons and diploids that differed both in growth form (sheets or small pellets of mycelium) and in the conditions of culture. This was not satisfactory, although in both cases wild-type controls were included. It is not known if it will be possible to grow heterokaryons of *A. nidulans* in large quantities in submerged culture without overgrowth by diploid strains, or what effect the numbers and types of nutritional marker used to balance the heterokaryons has on their growth. It is feasible that special genetic techniques could be developed to suppress the diploids. It may also be possible to find conditions of static culture which will permit growth of heterokaryotic mycelium (Cove, personal communication).

The main purpose of this paper is to show that non-complementation of the sorbitol mutants in balanced heterokaryons is a real and repeatable phenomenon. Discussion of models to account for the difference in complementation of the mutants combined in balanced heterokaryons or in heterozygous diploids is hampered by lack of critical evidence to decide between *intra-* or *inter*-genic complementation. The two possible interpretations are that *sb8* represents a gene within which at least three groups of complementing mutants occur, or the groups themselves represent three linked genes spanned by a deletion (*sb8*). The analysis of crosses between the mutants showed that they were closely linked and favoured the second interpretation; that *sb8* is a deletion spanning three closely linked genes (*sb3*, *sb4*, *sb6*). But as the crosses involved complementary mutants and no linked markers were available, alternative interpretations of the 'apparent' recombinants cannot be ruled out, and this must remain a tentative conclusion. It is, however, supported by two other observations. First, the phenotypic difference between the total mutants (which constitute one of the complementation groups) and the leaky mutants in their ability to oxidize sorbitol. Second, the absence from the complementation map of a group of mutants non-complementary to two of the three groups (Fig. 1). The number of mutants tested was small but mutants of this type would be expected if this were *intra*-genic complementation (Catcheside, 1960). The paradox that the supposed deletion (*sb8*) has a leaky phenotype although including a gene (*sb3*) which determines a total mutant, is resolved if leaky growth is due to utilization of sorbitol by a second pathway, and growth of the total mutants is inhibited by the accumulation, on solid media, of an intermediate in sorbitol metabolism. Recent work by the author has shown that although the leaky mutants yielded normal growth on sorbitol + glycerol (which does not repress the sorbitol oxidation system) the total mutants failed to grow. Both leaky and total

mutants yielded normal wild-type growth on sorbitol + glucose (which represses the sorbitol oxidation system) and on glycerol or glucose + glycerol. Dilution of the inhibitory intermediate probably prevented detection of this effect in the induction experiments in liqud culture.

It is reasonable to assume that the difference shown in the utilization and oxidation of sorbitol by mutants combined in heterokaryons or in diploids results from a difference in enzyme formation rather than function in the two systems, but this cannot be proved until the enzymes of sorbitol metabolism are identified and the precise metabolic lesions in the mutants established. However, it appears that in this case genes which are probably not allelic, are able to complement and form an active enzyme system in heterozygous diploids—where they are on different chromosomes in the same nucleus—but cannot complement or form the active enzyme system in balanced heterokaryons—where they are in different nuclei. Two types of general model may be suggested to account for this. First, gene products cannot interact across the cytoplasm; second, a regulatory signal transmitted by one nucleus does not arrive at its receiver in the other nucleus (Jacob & Monod 1961).

The simplest way in which gene products could fail to interact is that they do not reach critical local or relative concentrations in heterokaryons because they are diffusing from different points, whereas these critical concentrations are attained in diploids where the products are diffusing from the same points. Such a model could be considered in the case of *intra*-genic complementation, when the interacting components may be similar polypeptide chains (Fincham, 1962; Fincham & Coddington, 1963; Partridge, 1960; Loper, 1961), or *inter*-genic complementation when the components may be dissimilar polypeptides (Yanofsky, 1960; Shaw & Barto, 1963). It has also been suggested that *intra*-genic complementation may only occur in diploids as the result of an interaction of gene products at a stage of synthesis intermediate between DNA and polypeptide (Catcheside, 1960; Dorn & Burdick, 1962).

It is tempting to attempt to account for the present results using the Jacob and Monod model for the regulation of protein synthesis (Jacob & Monod, 1961), particularly as the enzyme system is inducible. However, a fundamental difficulty arises since the essential feature of this model is a negative action of the repressor substance on the operator site and the presence of regulator and operator genes in the same nucleus may be expected more effective in repressing enzyme formation than when they are separated in different nuclei. The present case seems to require a positive interaction, and is, in this respect, similar to that of alkaline phosphatase in *Escherichia coli* where there is evidence for an endogenous inducer in the regulatory mechanism (Garen & Echols, 1962).

Note added after acceptance for publication

A paper by Pontecorvo has recently appeared (*Proc. roy. Soc. B*, **158**, 1, 1963) in which he modifies the Jacob and Monod model and advances the hypothesis that the product of regulator genes are confined to the nuclei of higher organisms. A number of new examples are given in which unlinked genes yield mutant phenotypes when

combined in balanced hetero-karyons of *Aspergillus* or dikaryons of *Coprinus* (the work of D. Lewis), but wild phenotypes when combined in heterozygous diploids of these organisms. These results are persuasively explained by a model of 'cascade regulation' in which the activity of the regulator gene (R_1) that controls a particular structural gene (S) is itself controlled by a second-order regulator gene (R_2). The repressors formed by each of the regulator genes are supposed not to escape from the nuclei and therefore the rates of transcription of some structural genes may be different in heterokaryons or in diploids. Two of the new examples are like the present case, recessive non-allelic genes complement in diploids but not in heterokaryons. Pontecorvo explains this by equating one gene with R_2 and the other with S. For complementation to occur R_2^+ and S^+ must be in the same nucleus since the repressor formed by R_2^+ cannot act across the cytoplasm.

The 'cascade model' presents a rationale for positive interactions between genes which could not be achieved with the Jacob and Monod model. It can be applied to the present case with a generally satisfactory result although one point is not accounted for. It is assumed, as before, that there are two paths for sorbitol metabolism in *Aspergillus*, an oxidative and a non-oxidative. The leaky mutants are defective in the oxidative enzyme (A) and grow by the non-oxidative path, as does the deletion (*sb8*). The total mutants are blocked in an enzyme (B) later in the oxidative pathway and accumulate a toxic intermediate. Using the 'cascade model' for the three complementing groups of mutants, R_2 or S_A = *sb4* or *sb6* and S_B = *sb3*. Sorbitol antagonizes the repressor formed by R_1, and glucose, or a metabolic product of glucose, that formed by R_2. The genes R_2^+ and S_A^+ or R_2^+ and S_B^+ will not complement in heterokaryons if the repressor formed by R_2^+ is restricted to the nucleus. This model does not account for the non-complementation of S_A^+ and S_B^+ in heterokaryons.

5. SUMMARY

1. Two total and five leaky sorbitol mutants isolated in *Aspergillus nidulans* by defective growth on the sugar are all recessive. The mutants are closely linked, they appear to represent three linked genes spanned by a deletion.

2. Mutants which complement in heterozygous diploids do not complement in balanced heterokaryons. Failure to complement is a property of the mutants and not the result of a nutritional interaction or an unfavourable nuclear ratio in the heterokaryons.

3. Sorbitol is oxidized by an inducible enzyme system in the wild-type. There are at least two enzymes concerned in the oxidative assimilation of sorbitol, an initial oxidative enzyme, which is defective in the leaky mutants, and a later enzyme defective in the total mutants. There may also be a second non-oxidative pathway for sorbitol metabolism.

4. In diploids complementary pairs of mutants oxidized sorbitol at 75% the rate of the wild-type but non-complementary mutants did not oxidize the sugar. In balanced heterokaryons none of the pairs of mutants oxidized the substrate. It is concluded that failure of *inter*-genic complementation in the heterokaryons is the result of a

failure of either enzyme formation or enzyme function. Models to account for differences in enzyme formation in heterokaryons and diploids are suggested.

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