STUDIES OF ADENYLOSUCCINASE IN MUTANTS AND REVERTANTS OF NEUROSPORA CRASSA[1]

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MANY investigations of mutation in both forward and reverse directions have been made during the past three decades (see Brookhaven Symposia in Biology, 1955). Most of the earlier experiments were performed with Drosophila (PATTERSON and MULLER 1930; TIMOFÉEFF-RESSOVSKY 1933; KAUFMANN 1942; LEFEVRE 1950) or maize (STADLER 1944; STADLER and ROMAN 1948) and utilized gross morphological phenotypes as criteria for determining whether or not a particular gene had undergone complete reverse mutation. More recently, investigations have been made employing nutritional mutants of microorganisms (RYAN and LEDERBERG 1946; GILES and LEDERBERG 1948; KØLMARK and WESTERGAARD 1949; GILES 1951, 1956; GILES, DE SERRES and PARTRIDGE 1955; GLOVER 1956; DE SERRES 1958). Nutritional mutants permit the detection of certain intermediate mutational states at specific loci by means of accumulated intermediates, differential growth rates, etc. Where the genetic control over specific enzymes is known, measurements of enzyme activity in revertants have provided a means of distinguishing among revertants which are phenotypically similar in their growth rates on minimal medium. In a study of 15 ultraviolet-induced reverse mutants from an *ad-4* (adenylosuccinaseless) mutant in Neurospora (GILES, PARTRIDGE and NELSON 1957), all revertants were found to have a level of adenylosuccinase activity less than that of the wild type. Quantitative measurements of glutamic dehydrogenase activity in back mutants derived from one of the *am* (glutamic acid dehydrogenase deficient) alleles in Neurospora (PATEMAN 1957) revealed that seven of 28 back mutants possessed less enzyme activity than the original wild type. The assay procedure, however, did not permit a distinction among revertants having subnormal levels of enzyme activity nor among any of those apparently equivalent to wild type. Recent study of tryptophan-independent revertants in *E. coli*, derived from mutants lacking tryptophan synthetase activity (STADLER and YANOFSKY 1959; YANOFSKY and CRAWFORD 1959), shows that both suppressor mutation and reverse mutation occur. Qualitative as well as quantitative distinctions at the enzyme level were made between partial revertants and wild type.

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Previous reports have indicated the complexity of the *ad-4* locus, which controls the synthesis of adenylosuccinase in Neurospora (GILES, PARTRIDGE and NELSON 1957; WOODWARD 1958; GILES 1958; WOODWARD, PARTRIDGE and GILES 1958; WOODWARD 1959). The present study represents an attempt to analyze several mutant phenotypes at the enzyme level. From this study, it is apparent that enzyme differences may result from either forward or reverse mutational changes at this locus.

Terminology

The terms reversion and revertant are used in this paper to indicate any adenine-independent isolate arising from an *ad-4* mutant (GILES 1951). When reverse mutation is specified, genetic tests have indicated a change at or very near the *ad-4* locus. By this definition, all *ad-4* revertants analyzed genetically to date have resulted from reverse mutation rather than from suppressor mutation.

Primary mutants are mutants derived directly from wild type, and primary revertants are revertants obtained directly from primary mutants. Similarly, secondary mutants are derived from primary revertants and secondary revertants, from secondary mutants.

Genetic markers utilized in this study were: *leu* (*leu-1*), a leucine requiring mutant; *leu*$^+$, a leucine-independent allele of *leu-1*; *pan-2*, a pantothenic acid requiring mutant.

MATERIALS AND METHODS

All Neurospora mutants used in this investigation were obtained from a 74A St. Lawrence wild type stock or from strains that were derived by inbreeding of 74A. Mutant F23 is of spontaneous origin; all other primary mutants were isolated from macroconidia treated with 36,000r of X-rays. The assay procedure for making adenylosuccinase activity determinations (spectrophotometric measurement of the disappearance of adenosine monophosphate succinate [AMP-S]) has been described (GILES, PARTRIDGE and NELSON 1957). This procedure was modified in the present experiments by the substitution of Tris (tris [hydroxymethyl] aminomethane) HCl for phosphate buffer except where otherwise noted. All assays were performed at 35.5°C.

Reverse mutation experiments were performed with double mutants (*pan-2 ad-4*) in all cases. *ad-4* revertants were isolated by plating heavy suspensions of irradiated or nonirradiated conidia from such double mutants onto a minimal medium containing pantothenic acid. This procedure eliminates to a considerable extent the possibility of recovering undetected contaminant wild types and screens selectively for reversions to adenine independence. Only *ad-4*$^+$ colonies carrying the *pan-2* marker were saved, and these were routinely backcrossed to the parental *ad-4* mutant to obtain homocaryotic *ad-4*$^+$ *pan-2* strains. Single ascospore isolates were then used for subsequent enzyme assays. Assays of a given revertant with or without the *pan* marker agree as well as replicate assays of a culture from a single spore isolate. Revertants obtained from secondary mutant Y203-M1 were not backcrossed. In this instance, revertants were assayed directly

for adenylosuccinase activity because backcrossing difficulties were encountered; presumably most such revertants were heterocaryotic.

Primary and secondary *ad-4* mutants (WOODWARD, PARTRIDGE and GILES 1958) were obtained by the filtration and selective plating method (WOODWARD, DEZEEUW and SRB 1954). It was not necessary initially to test the growth response of all the colonies isolated, since *ad-4* mutants are distinguishable by the accumulation of a slight amount of purple pigment in the conidia when adenine in the medium is limiting.

RESULTS

Quantitative studies of adenylosuccinase in revertants: In this investigation, quantitative levels of adenylosuccinase activity were measured in extracts of a large number of revertants obtained from eight different *ad-4* alleles. Five of the eight alleles are shown in the complementation map of the *ad-4* locus (Figure 1),

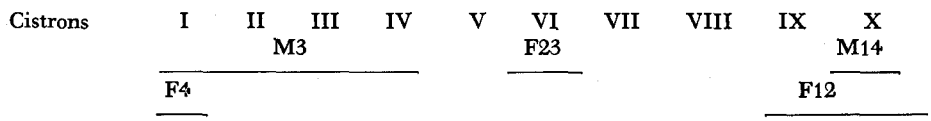

FIGURE 1.—Complementation map of the *ad-4* locus showing the relationship that exists among the mutants used in this investigation. Mutants F2, F20 and M1 are noncomplementing types, hence are not shown here.

which illustrates the pattern of complementation characteristic of each allele (WOODWARD, PARTRIDGE and GILES 1958). The other three alleles are noncomplementing mutants.

The degree of restoration of adenylosuccinase activity in the revertants varies, and appears to depend primarily on the allele from which each was obtained. The frequency distribution of activities in revertants is not a continuous one, however, since among the 112 revertants examined, only a limited number of fairly discrete quantitative categories are represented (Figure 2). The diagram in Figure 3 (together with Table 1 and Figure 2) indicates the origin and relationships of primary and secondary revertants used in this investigation. For example, mutant F12 has yielded 38 revertants with approximately 25 percent of wild type enzyme activity, nine with three percent of wild type activity, and one revertant with ten percent of wild type activity. In most cases, the same quantitative categories are observed among revertants of spontaneous origin as among ultraviolet- or X-ray-induced revertants (Table 1). To insure independence of origin for F12 revertants of spontaneous origin, each spontaneous revertant was isolated from a separate mutant culture.

The only class of revertants characterized by growth rates less than that of wild type on an adenine-free medium is the group exhibiting only three percent of wild type enzyme activity. These revertants show only slightly subnormal growth rates, but the reality of these differences can be established by the increases in growth rates observed upon supplementation with adenine (Table 2).

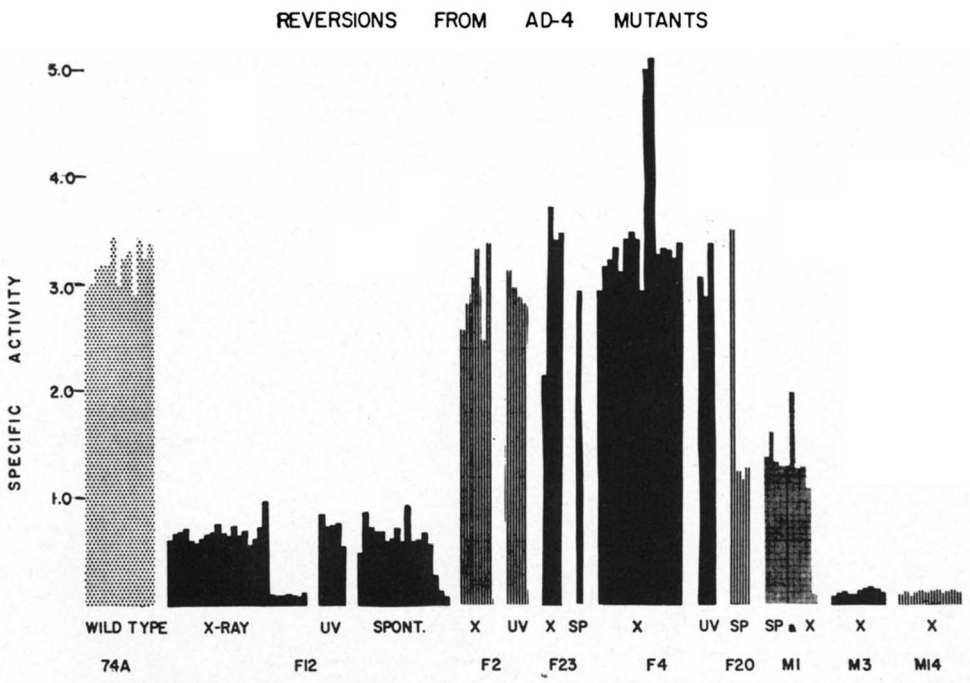

FIGURE 2.—Adenylosuccinase activities in revertants of independent origin. The eight *ad-4* alleles used yielded revertants spontaneously and/or following X-ray or ultraviolet irradiation. Specific activity was measured as $\Delta A_{280}/2.8$ mg dry wt/20 min/0.77 ml (see Table 3 for full details). Common parental mutant (in bottom line) for a revertant group is indicated by common marking pattern of bars representing that group.

Two primary reverse mutants from F12 gave rise to several secondary mutants. The primary reverse mutant having three percent of wild type enzyme activity was used to produce two secondary mutants (M3 and M14), from which 20 secondary revertants were derived. All of these possessed approximately three percent of wild type activity. On the other hand, the primary F12 reverse mutant having 25 percent of wild type enzyme activity gave rise (through secondary mutant M1) to secondary revertants possessing a higher level of enzyme activity than their parental primary reverse mutant, as well as to others in the three percent category (Figures 2 and 3).

Genetic studies of revertants: Previous reverse mutation studies at the *ad-4* locus showed that only four of 21 mutants tested failed to yield adenine-independent colonies following mutagenic treatment (NELSON 1957). None of the revertants investigated appeared to involve suppressor mutations as judged by an analysis of crosses in which loosely linked or nonlinked suppressors would have been detected (GILES 1958). The mutants that do revert to adenine independence appear to have characteristic reverse mutation rates. Such differences in reverse mutation rates are probably allele specific and presumably indicate that mutability isoalleles are present at the *ad-4* locus, as has been shown to be true at other

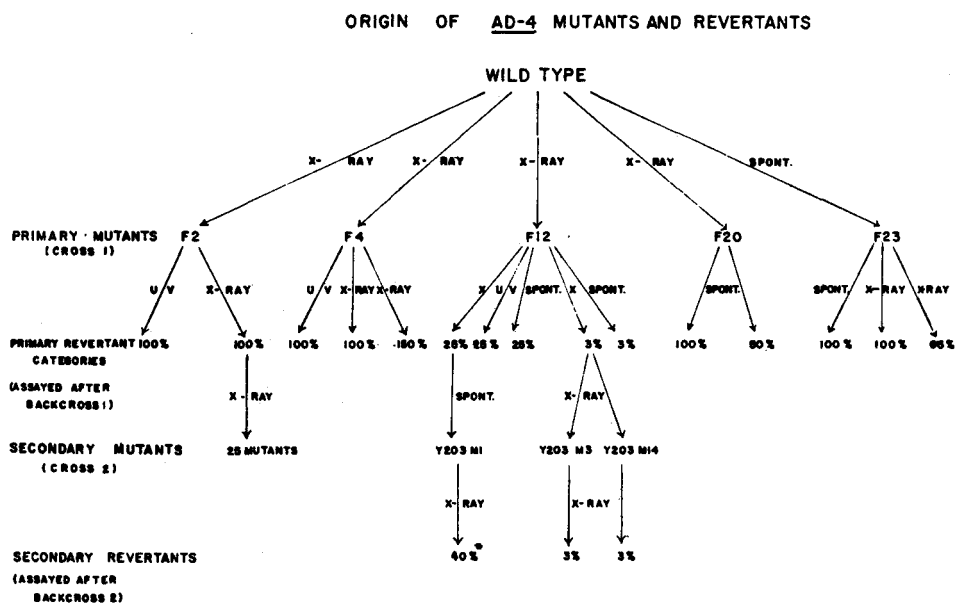

FIGURE 3.—Derivation of primary and secondary revertants. All *ad-4* mutants were crossed (crosses 1 and 2) to a *pan-2* mutant (unlinked marker). Double mutants (*ad-4 pan-2*) were used for the production of revertants in all cases. All primary and most secondary revertants were backcrossed (backcrosses 1 and 2) to their parental *ad-4* mutants to obtain homocaryotic stocks. Percentage figures indicate the approximate specific adenylosuccinase activities of the quantitative categories of revertants obtained, expressed as percent of wild type activity.

* Revertants from Y203 M1 were not backcrossed before assay.

loci in Neurospora (GILES 1951; GILES, DE SERRES, and PARTRIDGE 1955). However, definitive tests employing allelic crosses having linked markers have not yet been performed.

Genetic information has been obtained from crosses of revertants selected from different quantitative categories with respect to adenylosuccinase activity. In general, the results of such crosses, employing linked markers, indicate that the quantitative differences in adenylosuccinase activities characteristic of individual revertants are inherited as allelic differences at the *ad-4* locus (GILES 1958). Additional data supporting this view are presented in Tables 3 and 4. The relatively high activity values (averaging about 50 percent) for the two F12 revertants indicated in Table 4 are similar to results obtained in previous studies employing mycelial extracts prepared in phosphate buffer (GILES, PARTRIDGE, and NELSON 1957). This effect of the extraction buffer on adenylosuccinase activity will be discussed further in the next section of this paper.

The present results, together with those presented previously from both ordered and random ascospore isolations from crosses of *ad-4* revertants with wild type (GILES 1958), provide evidence that substantially all *ad-4* reversions arise by reverse mutation rather than by suppressor mutation. In addition, the tetrad data from crosses of revertants having subnormal levels of adenylosuccinase ac-

TABLE 1

Classification of ad-4 *revertants according to their adenylosuccinase activity relative to that of wild type (74A)*

Primary mutants	Pattern of complementation	Mutagen used in obtaining revertants	Quantitative categories represented in percent	No. rev./ category
F2	none	X-ray	100	6
		U. V.	100	4
F4	Cistron I	X-ray	100	7
		X-ray	150	2
		U. V.	100	3
F12	Cistrons IX and X	X-ray	3	7
		X-ray	25	20
		U. V.	25	5
		none	3	2
		none	10	1
		none	25	14
F20	none	none	35	3
		none	100	1
F23	Cistron VI	X-ray	65	1
		X-ray	100	3
		none	100	1
Secondary mutants	(derived from F12 revertants)			
M14	Cistron X	X-ray	3	12
M3	Cistrons I, II, III and IV	X-ray	3	10
M1*	none	X-ray	3	1
		X-ray	35	9

See Figure 1 for complementation map of mutants used to obtain revertants. See Figure 2 for individual revertant activities.

* Revertants from this mutant were not backcrossed to obtain homocaryotic single spore isolates due to crossing difficulties.

tivity indicate that modifying factors at other loci are of relatively minor importance in affecting the levels of enzyme activity in various revertants.

Only two cases have been found in which the quantitative level of enzyme activity appears to involve modifiers at other loci. One of these is a primary revertant of F4, Y201-R24, which possesses 150 percent of wild type adenylosuccinase activity. Table 5 shows the segregation of high enzyme activity in what appears to be a random fashion with respect to the *leu-1* marker which has been mapped about two units to the right of the *ad-4* locus (NELSON 1957). The other revertant that seems to be influenced by modifiers was derived from mutant F12. In this instance, only three cultures obtained from random spore isolations have been assayed. These assays gave the widely differing values of 12.7, 15.8, and 21.0 percent of wild type enzyme activity. In all other revertants that were studied, the results from separate spore cultures agree as closely as separately grown cultures of the same isolate (see Table 3).

Complementation studies with reverse mutants: Attempts have been made to detect complementation between reverse mutants (derived from either the same or different alleles) having subnormal levels of enzyme activity. In no case was a synergism or inhibition detected; the heterocaryons merely showed an additive effect of the two separate enzyme activities. These heterocaryons were forced by using nutritional mutant markers which would not permit growth on the minimal medium without heterocaryosis. It has been possible, however, to detect complementation between revertants having three percent of wild type activity and certain complete mutants (WOODWARD 1959). Such complementation resulted in a level of enzyme activity in the heterocaryon approximating 25 percent of wild type.

Qualitative differences between enzymes from reverse mutants and wild type: Preliminary tests indicate that there are qualitative differences associated with the enzymes extracted from reverse mutants having subnormal levels of adenylosuccinase activity. Furthermore, the tests that have been made indicate that all of the revertants from a single quantitative category contain an AMP-S splitting enzyme with similar properties. For example, the revertants with three percent of wild type activity are also characterized by a lag of five minutes or longer before the maximal rate of reaction is attained. A similar lag is observed among revertants with 25 percent of wild type activity, but in addition, the specific activity of revertants in this category is increased to approximately 50 percent of wild type by extracting in pH 7.0 phosphate buffer instead of the usual pH 8.0 Tris HCl

TABLE 2

Linear growth rates of F12 revertants at 25°C on supplemented and unsupplemented agar medium (in horizontal test tubes)

Revertant number	Approximate adenylosuccinase activity (percent of W.T.)	Growth in mm/hr over 30 hr period		
		Minimal	150 μg/ml adenine	Difference
74A wild type	100	3.94	4.00	+0.06
3	25	3.59	3.62	+0.03
4	25	3.68	3.62	−0.06
9	25	3.75	3.88	+0.13
15	25	3.78	3.84	+0.06
18	25	3.47	3.45	−0.02
19	25	3.70	3.68	−0.02
25	25	4.00	3.91	−0.09
26	25	3.94	3.94	0.00
10	3	3.23	3.68	+0.45
11	3	2.49	2.64	+0.15
13	3	3.13	3.68	+0.55
17	3	3.78	4.06	+0.28
20	3	3.15	3.47	+0.32
24	3	3.43	4.00	+0.57
28	3	3.02	3.83	+0.81
30	3	3.37	3.91	+0.54

TABLE 3

Segregation of subnormal levels of adenylosuccinase activity

Cross	Phenotype	Ascus and spore no.	Relative activity*	Percent of wild type activity
Y201–R9 × *leu-1*	leu	3.1	3.39	105.5
	leu	3.3	3.46	108.0
	leu$^+$	3.5	1.43	44.5
	leu$^+$	3.7	1.34	42.0
Y191–R14 × *leu-1*	leu$^+$	1.1	0.78	24.5
	leu$^+$	1.2	0.85	26.5
	leu	1.3	4.24	132.5
	leu	1.4	4.12	130.0
	leu$^+$	1.5	0.71	22.0
	leu$^+$	1.6	0.80	25.0
	leu	1.7	3.88	121.0
Y191–R28 × *leu-1*	leu	2.1	2.72	85.0
	leu	2.2	3.20	100.0
	leu	2.3	3.26	102.0
	leu	2.4	3.02	94.5
	leu$^+$	2.5	0.19	5.9
	leu$^+$	2.6	0.14	4.4
	leu$^+$	2.7	0.16	5.0
	leu$^+$	2.8	0.15	4.7
Y191–R14 × Y191–R13	leu$^+$	4.1	0.16	5.0
	leu$^+$	4.2	0.16	5.0
	leu$^+$	4.3	0.74	23.0
	leu$^+$	4.4	0.76	23.5
	leu$^+$	4.5	0.77	24.0
	leu$^+$	4.6	0.76	23.5
	leu$^+$	4.7	0.15	4.7
	leu$^+$	4.8	0.14	4.4

* Measured as ΔA_{280}/20 min/2.8 mg dry wt/0.77 ml total volume with excess substrate at 35.5°C in pH 8.4, 0.05 *M* Tris HCl; extracted in pH 8.0, 0.05 *M* Tris HCl after three days growth at 25°C (Standard conditions).

extraction buffer (Table 6). (Tris buffer of pH 7.0, employed as a control in this experiment, eliminated pH as the factor responsible for the large differences observed.) Furthermore the lag in the reaction observed when the enzyme is extracted in Tris buffer does not appear when the enzyme is extracted in phosphate. The lag is not eliminated by the addition to the Tris-buffer-extracted enzyme of pyrophosphate, phosphate, sodium or potassium, or combinations of these. No differences in specific activity have been observed when other classes of revertants or the wild type strain have been extracted in the two different buffers.

An F23 revertant with about 65 percent of wild type enzyme activity can be distinguished from wild type by comparing reaction rate (decrease in absorption) *vs.* time (Figure 4). The revertant enzyme requires about 3.5 minutes to attain its maximum reaction rate and the rate begins to decrease at a higher residual substrate concentration than is the case with the wild type enzyme.

One of the revertants of F12, in the 25 percent activity class, (Y 191-R4), was subjected to further qualitative tests. It had been noted in a preliminary survey that this was one of the few strains which produced an enzyme differing in substrate specificity from the wild type (GILES 1958). Therefore, other conditions were sought which would further characterize it qualitatively. Some of the same tests were also carried out on another similar revertant of F12 (Y219-R1) and on revertant progeny of R4. In general, it appears that the revertant enzymes are

TABLE 4

Adenylosuccinase activity of segregants from crosses of UV-induced back mutants F12–R164 and F12–R165 with a leucine-requiring strain (leu-1)

Expt. no.	Back mutant parent	Ascus and spore no.	Phenotype	Relative activity* (percent leucine parent)
1	R164	1.1	leu$^+$	50
		1.4	leu$^+$	50
		1.6	leu	88
		1.8	leu	102
	R164	5.1	leu	80
		5.4	leu$^+$	50
		5.5	leu$^+$	40
		5.7	leu	86
	R165	41.1	leu	78
		41.4	leu	82
		41.5	leu$^+$	40
		41.7	leu$^+$	44
	R165	43.2	leu	98
		43.4	leu$^+$	38
		43.5	leu$^+$	31
		43.8	leu	77
	Controls:	630–2a (*leu-1*)	leu	98
		630–1A (*leu-1*)	leu	102
		R164	leu$^+$	52
		R165	leu$^+$	47
2	R164	5.1	leu	121
		5.4	leu$^+$	51
		5.5	leu$^+$	58
		5.7	leu	103
	R165	42.2	leu	93
		42.4	leu	108
		42.5	leu$^+$	58
		42.8	leu$^+$	50
	Controls:	630–2a (*leu-1*)	leu	100
		630–1A (*leu-1*)	leu	89
		R164	leu$^+$	61

* Enzyme activity measured with excess substrate at 35.5°C in pH 8.4, 0.05 *M* Tris HCl; 1:11 dilution of pH 7.0 0.05 *M* phosphate extract of 40 mg mycelium/ml; 69 hours growth at 25°C.

TABLE 5

Segregation of high adenylosuccinase activity

Cross	Phenotype	Ascus and spore	Relative activity*	Percent of wild type activity
Y201–R24 × *leu-1*	leu$^+$	3.1	2.86	91.0
	leu$^+$	3.3	4.25	133.0
	leu	3.5	3.02	94.5
	leu	3.7	7.15	223.0
	leu$^+$	5.2	3.76	118.0
	leu	5.4	3.32	104.0
	leu$^+$	5.6	4.36	136.5
	leu	5.8	3.48	109.0
	leu	6.1	....	....
	leu$^+$	6.3	2.67	83.5
	leu$^+$	6.5	4.32	135.0
	leu	6.7	3.18	99.5

* Standard conditions (see Table 3).

more sensitive to inhibitory or deleterious treatments than is the wild type enzyme. Furthermore these differences are greater when the substrate used is SAICAR (N-[-5-amino-1-ribosyl-4-imidazolecarbonyl]-L-aspartic acid 5′-phosphate) than when it is AMP-S, as will be discussed later.

The revertant enzymes are much more thermolabile than is wild type enzyme; incubation at 35°C for seven minutes, while having no effect on wild type enzyme, destroys 90 to 100 percent of the activity of the revertants. However, this sensitivity disappears in the presence of glutathione (10^{-3}M). In fact, glutathione (with or without preincubation) increases the rate of AMP-S splitting by R4 by 33 percent, while having little effect on wild type activity. (The spectral changes in the course of the reaction were not altered qualitatively by the addition of glutathione).

Revertant activity is also inhibited by zinc at a concentration much lower than that affecting the wild type. R4 was chosen for further examination of the influence of metal ions and a procedure was adopted involving a 7.25 minute preincubation of the extract with the chloride salt of the metal in the presence of 10^{-3} molar glutathione, before addition of substrate. Constant maximal reaction rates were compared with controls identical except for the omission of metal salts. At the 10^{-5} molar level, divalent zinc and copper both inhibit the splitting of AMP-S by 65 percent, while divalent nickel and cobalt and trivalent chromium and iron produce little or no inhibition. Wild type is not inhibited by either copper or zinc at these levels. An identical test of R4 action on SAICAR showed a similar zinc inhibition (71 percent), again with no inhibition of wild type.

In a series of four asci from two crosses, the pairs of R4 progeny (carrying parental markers) which gave the highest and lowest activities on both AMP-S and SAICAR (cf. GILES 1958) were tested (as above) for zinc inhibition on

AMP-S. The most active pair was least inhibited (by 65 percent) and the least active pair was most inhibited (by 85 percent) by 10^{-5} molar zinc (Table 7). In all these progeny specific activities for both substrates were invariably much closer to those of the parent carrying the same genetic marker than to those of the other parent, indicating the allele specificity of the enzymic activity.

Comparisons of substrate specificities: When the existence, in the same biosynthetic sequence, of a second natural substrate for the single enzyme, adenylosuccinase, was discovered (BUCHANAN *et al.* 1957; GILES, PARTRIDGE, and NELSON 1957; GOTS and GOLUB 1957), tests were made on representative complementary *ad-4* mutants to determine whether their behavior might be based on differences

TABLE 6

Comparison of adenylosuccinase activities of representative revertants with Tris vs. *phosphate extraction buffers*

Strain	Relative activity*	Extraction buffer	pH	Percent of W.T. activity
74A (wild type)	3.16	Tris HCl	8.0	100.0
	3.10	Phosphate	7.0	100.0
F12–Y191–R19	0.98	Tris HCl	8.0	31.0
	1.46	Phosphate	7.0	47.2
	1.10	Tris HCl	7.0	34.3
F12–Y167–R168	0.73	Tris HCl	8.0	23.1
	1.74	Phosphate	7.0	56.2
F12–Y167–R166	0.85	Tris HCl	8.0	27.4
	1.74	Phosphate	7.0	56.2
F12–Y167–R184	0.74	Tris HCl	8.0	23.4
	1.63	Phosphate	7.0	52.6
F12–Y167–R186	0.76	Tris HCl	8.0	24.0
	1.42	Phosphate	7.0	45.8
F12–Y167–R156	0.54	Tris HCl	8.0	17.1
	1.46	Phosphate	7.0	47.2
F12–Y191–R28	0.09	Tris HCl	8.0	2.85
	0.065	Phosphate	7.0	2.10
F4–Y164–R128	3.48	Tris HCl	8.0	111.0
	3.34	Phosphate	7.0	107.5
F2–Y164–R15	3.04	Tris HCl	8.0	96.3
	3.30	Phosphate	7.0	106.5
F12–Y209–R1	0.79	Tris HCl	8.0	24.7
	1.03	Tris HCl	7.0	32.2
F4–Y201–R23	3.36	Tris HCl	8.0	105.0
	3.24	Tris HCl	7.0	101.0

* Standard conditions (see Table 3).

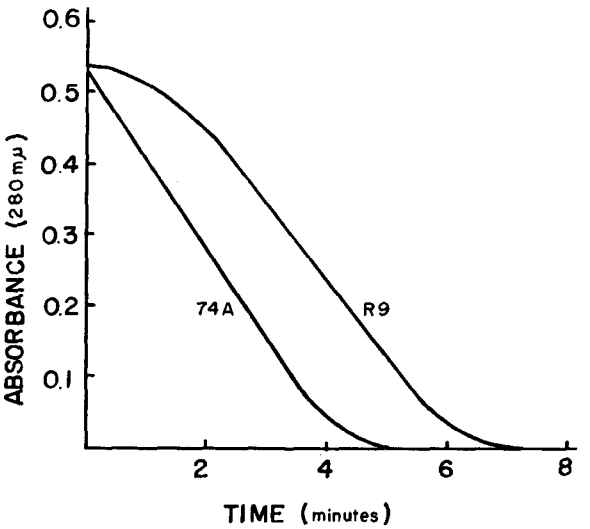

FIGURE 4.—Continuous tracing of the decrease in absorbance at 280 mμ. The wild type (74A) extract was diluted to give approximately the same maximal rate of decrease in absorbance as the extract from F23 revertant Y210–R9.

TABLE 7

Adenylosuccinase activity of segregants from crosses of reverse mutant Y191-R4 with a leucine requiring strain (leu-1) *and with its parental mutant (F12)*

Cross no.	Parents	Ascus and spore no.	Phenotype	Substrate: AMP-S Relative activity (percent W.T.)	Substrate: SAICAR Relative activity (percent W.T.)	Substrate: AMP-S (+ 10^{-3} *M* glutathione ± 10^{-5} *M* $ZnCl_2$) percent inhibition by $ZnCl_2$
1	R4 × *leu-1*	9.1	leu	100	119	. .
		9.4	leu	99	160	. .
		9.5	leu$^+$	21	15	62
		9.7	leu$^+$	22	15	67
		10.1	leu$^+$	21	14	. .
		10.4	leu$^+$	20	13	. .
		10.5	leu	98	119	. .
		10.7	leu	110	132	. .
2	R4 × F12	2.1	ad$^+$	18	6	78
		2.4	ad$^+$	20	6	88
		3.1	ad$^+$	22	9	. .
		3.4	ad$^+$	18	7	. .
	R4	. .	ad$^+$	23	7	62
	leu-1	. .	leu	100	112	. .

Standard conditions of growth, extraction and assay, except for addition in last column, as noted.

in the specificities of their activities for the two substrates AMP-S and SAICAR (GILES, PARTRIDGE, and NELSON 1957; GILES 1958). No such differences were found.

Additional observations have provided further evidence that adenylosuccinase is a single enzyme. Even in the case of a revertant possessing specific activities twice the normal level (derived from mutant F4), the relative substrate specificity was similar to that of wild type. Furthermore, in a chromatogram (on DEAE cellulose, with salt gradient elution) of a thermolabile adenylosuccinase (from the temperature-sensitive mutant 44206 = F15) the activity ratio for the two substrates remained constant throughout the series of active eluate fractions, as was the case in BUCHANAN's purification of the normal enzyme from yeast (MILLER, LUKENS, and BUCHANAN 1959). It was also observed that the enzyme produced by an interallelic heterocaryon (F4 + F23) showed a wild type ratio of activities for the two substrates and was no more sensitive to zinc than was the normal enzyme, even after chromatographic purification.

However, despite the fact that most strains are not distinguishable from wild type in substrate specificity, when a number of revertants were examined, several were found to possess definitely less activity for SAICAR than for AMP-S (expressed in percentage of wild type specific activity). One of these (R4) was selected for further study; although certain preliminary results of this study were reported previously (GILES 1958), these observations have now been extended further with the results reported here.

Under ordinary assay conditions the revertant enzyme splits SAICAR at a relatively much slower rate than AMP-S, but a constant velocity is maintained from the beginning of the reaction, without the lag or activation period observed on AMP-S. The addition of glutathione eliminates these differences. That is, the reaction velocity of SAICAR increases to a constant value, after about 20 minutes, which is 245 percent greater than its rate without glutathione. The AMP-S reaction rate is increased by only 33 percent by glutathione (or by 2, 3-dimercaptopropanol). Thus, activation of the revertant enzyme with glutathione can greatly reduce if not eliminate the differential in relative reaction velocities of the two substrates, in addition to removing their differences in substrate activation ability. It has also been observed (using derived R4 strains) that a twofold increase in initial AMP-S concentration over the standard level doubles the constant maximum reaction velocity attained after the usual lag period. This substrate activation effect is rapidly reversible, as evidenced by a renewal of the lag on addition of more substrate after the end of the period of constant velocity.

In a further attempt to determine whether mutational changes can produce *ad-4* alleles with clear differences in relative substrate specificities, a procedure has been devised to detect revertants having the ability to split AMP-S but not SAICAR. This procedure involves plating ultraviolet- or X-ray-treated conidia of various *ad-4* mutants on a medium—minimal supplemented with hypoxanthine—which does not support the growth of the original mutants. Colonies which appear are tested for growth on unsupplemented medium, in a search for mutants which can carry out the later but not the earlier biosynthetic function of adeny-

losuccinase. Among 115 isolates tested, from two different *ad-4* mutants, no such differential revertants were found. That is, none would grow on hypoxanthine without growing on minimal medium also.

DISCUSSION

The existence of complete reverse mutation has been viewed with some skepticism (GOLDSCHMIDT 1955). Complete reverse or return mutation is taken to mean that the mutant gene in question has been changed back to a structure in every way identical with its original configuration. To decide this matter definitely would require the elucidation of the precise molecular structure of a given gene. A much less precise but, at present, more practical substitute would appear to be an examination and comparison of gene products, such as a particular enzyme under the control of a given locus. The ultimate test, even at the gene product level would require a detailed structural chemical analysis; i.e., an amino acid sequential analysis, or the detection of differences in secondary or tertiary structure if other tests failed to distinguish between the enzymes produced by the reverse mutant and the wild type.

The approach described in the present paper involves a less definitive characterization of the gene product; nevertheless, distinctions can be made between the wild type enzyme and the enzymes produced by revertants having subnormal levels of enzyme activity. It appears significant that no qualitative difference has been detected between the wild type enzyme and the enzyme produced by reverse mutants having quantitative levels of enzyme activity equivalent to wild type (utilizing the same tests that permitted a distinction in the other cases). Studies on other enzymes (YURA 1959; SUSKIND, YANOFSKY and BONNER 1955) suggest that a subnormal level of enzyme activity may indicate only a qualitative rather than a quantitative difference in the enzyme in question.

The genetic data presented in this paper make it evident that most of the mutations studied—those occurring in both the forward and reverse directions—are associated with changes at the *ad-4* locus. On the basis that the mutational changes, as judged by alterations in the quantitative and/or qualitative measurements of the enzyme adenylosuccinase, are indeed reflections of changes in the molecular structure at the *ad-4* locus, the results of this investigation can be interpreted to indicate that a limited number of possible molecular configurations will result in repair of this locus, thus restoring adenylosuccinase activity. Some mutations lead to the formation of a protein which, although capable of carrying out the splitting reaction, differs in a quantitative and/or qualitative way from the wild type enzyme. The number of categories of revertants obtained varies with different mutant alleles. From F12, two distinct categories were recovered (3 percent and 25 percent) with a possible third category represented by the one revertant with ten percent of wild type enzyme activity. The fact that most of these quantitative categories are also separable on the basis of qualitative comparisons at the enzyme level supports the validity of the quantitative classification. Nonetheless, in spite of the similarities that are observed between enzymes

obtained from reverse mutants within a single quantitative category, the possibility exists that they may differ in more subtle ways that have not yet been detected. Such changes could reflect very small differences that are incapable of detection by the methods used, or they could be changes that are a matter of indifference to the enzyme as far as its activity is concerned. Actually, some evidence was found for qualitative differences in enzyme behavior between the "25 percent group" of revertants as a whole and at least one of its members, as will be discussed later. However, the fact that only a limited number of distinguishable types of reverse mutants has been obtained from a given allele suggests that only a limited number of changes can result in repair, either partial or complete, of the damage incurred at the time of the original forward mutational event.

Previous studies (NELSON 1957) have indicated that the damage incurred by forward (direct) mutation at the *ad-4* locus may be either reversible or irreversible, since certain alleles are apparently incapable of reverse mutation. In addition, the present studies have demonstrated that certain alleles, although capable of yielding revertants, apparently possess another type of irreversible damage, since they cannot undergo complete reverse mutation, as judged by the characteristics of the adenylosuccinase produced by revertants occurring in such mutants. In the case of the primary mutant, F12, only revertants with subnormal levels of enzyme activity have been obtained. Furthermore, a second type of irreversible change has apparently occurred in those primary revertants (from F12) with only three percent of wild type enzyme activity. This change is revealed when secondary mutants from three percent primary revertants are used to produce adenine-independent (secondary) revertants. Of 20 such secondary revertants assayed, all possessed approximately three percent of wild type enzyme activity. The 20 revertants assayed were selected from over 100 revertants on the basis of having the most rapid growth rates. On the other hand, a primary revertant (from F12) with 25 percent of wild type enzyme activity has been used to obtain secondary revertants possessing a higher level of activity than their parental primary revertant.

One F4 revertant having a high level of enzyme activity (150 percent of wild type) is interesting in that reverse mutation appears to have occurred at the *ad-4* locus in conjunction with an independent mutation at a loosely-linked or nonlinked locus. Both of these mutational changes seem to be involved in the attainment of the supernormal level of enzyme activity. When this modifier gene is crossed into wild type (employing crosses with markers closely linked to the *ad-4* locus), the modifier affects such a strain in a like manner; i.e., the level of enzyme activity is raised to over 150 percent of normal wild type. No qualitative differences have been detected between the enzymes from the revertant having a supernormal level of activity and from normal wild type. It may be noted in this connection that certain other strains possessing wild type *ad-4* alleles (*ad*$^+$), including some related to 74A and some unrelated strains, have yielded similarly high adenylosuccinase activities. Enzyme repressor genes of the type well-established in bacteria may be involved here.

Another instance of what may be modifier effects is seen (in Table 7) in the

quantitative and qualitative variations between revertant progeny in separate asci from crosses of a single revertant (R4). It therefore appears that factors in the genetic background as well as components of the assay medium (e.g. glutathione) may exert a marked influence on the degree of deviation of such revertants from normal in enzymatic assay behavior. These observations suggest the need for further evidence before concluding that indications of heterogeneity within a quantitative category of revertants (e.g., the relative substrate specificity within the 25 percent group) reflect real differences in the structure of the enzyme and its controlling locus. The possibility must be entertained that closely-linked modifiers may be responsible for altering the tertiary structure of the enzyme (during growth or during extraction) to different degrees in the various members of the quantitatively intermediate class of revertants. Whether these differences are expressed in the behavior of the enzymes in their native state *in vivo* remains problematical. At any rate, procedures devised for detecting absolute differences in substrate specificities of the enzyme during growth of induced revertants have not yet provided any evidence of such cases.

It seems probable that the degrees of difference in substrate specificity thus far observed *in vitro* are secondary reflections of differences in the condition of the enzyme molecules, as extracted. Such a situation may lead to varying degrees of reduction in the general efficiency and stability of enzyme molecules which are identical in their primary structure, these deviations being magnified in the presence of SAICAR as substrate as well as by generally unfavorable conditions during extraction and assay. The greater effect of AMP-S in activating the revertant (R4) enzyme, as compared with SAICAR (especially in the absence of added glutathione), may be connected with its more rigid structure and/or its possession of an extra carbon atom. This revertant form of adenylosuccinase may represent an extreme example of the requirement of enzymes in general for activation through alteration of surface configuration by contact with substrate, as KOSHLAND (1959) postulates.

In spite of these reservations concerning the interpretation of observed qualitative differences in the adenylosuccinase activity within one quantitative category of revertants, when the whole series of revertants is considered, the evidence clearly supports the attribution of the various qualitative and quantitative abnormalities in adenylosuccinase activity, characteristic of the different groups of revertants, to a variety of reverse mutational changes at the *ad-4* locus. Even within the restricted group just discussed, the differences have not been eliminated by crossing procedures, leaving the existence of associated allelic and enzymatic differences within major quantitative categories of revertants as the currently favored interpretation.

The present investigations are also of interest in connection with the question of whether reverse mutations can be induced by X-rays (cf. LEFEVRE 1950). Although certain *ad-4* alleles are apparently incapable of reverse mutation, the majority can revert either spontaneously, or following ultraviolet or X-ray treatment. Furthermore, there is no evidence for marked differences in the categories of reverse mutants induced in a given allele by various treatments. For certain

alleles (e.g., F2, F23, and F4), present evidence indicates that X-rays are capable of producing reverse mutants equivalent to the original wild type on the basis of both genetic analysis and enzyme assays. Thus, these results agree with evidence obtained at other loci in Neurospora (GILES 1951, 1956), in particular with those studies performed by DE SERRES (1958) with *ad-3* mutants. Recent experiments also indicate that reverse mutations can be induced by X-rays at certain loci in Drosophila (GREEN 1959).

Although these results indicate that X-rays can induce reverse mutation of certain *ad-4* alleles, there remains the related question of whether mutants initially produced by X-rays can back mutate. In the experiments reported here, general evidence supports the view that essentially all the *ad-4* mutants (including those capable of X-ray-induced reverse mutation) derived from macroconidia exposed to X-rays were indeed induced by the X-irradiation. This conclusion is supported by the fact that the yield of mutants was very much higher in irradiated conidia than in untreated conidia from parallel control experiments. Specific reconstruction experiments designed to eliminate the possibility that selection of pre-existing *ad-4* mutants might occur in the X-ray experiments were not performed. However, such experiments have been performed by DE SERRES (1958) with *ad-3* mutants and his results strongly support the view that adenine mutants derived from macroconidia exposed to X-rays and recovered by the filtration-concentration procedure have indeed been induced by the X-ray treatment.

SUMMARY

Revertants have been obtained from eight different *ad-4* (adenylosuccinaseless) mutants. Most of the mutants were derived from X-irradiated conidia, while revertants were obtained from untreated, X-irradiated, or ultraviolet-treated condia. Adenylosuccinase activity has been restored to at least some degree in all revertants. All present evidence indicates that the revertants studied are true back mutants. Suppressor mutations affecting the *ad-4* locus have not been found.

Reverse mutants derived from a given allele can be categorized according to the level of restored adenylosuccinase activity. There appear to be a limited number of quantitative levels of enzyme activity to which a given allele is capable of being restored by reverse mutation. Evidence is presented for certain qualitative differences in the enzymes obtained from revertants of different quantitative categories, while such differences have not been detected between enzymes from revertants within a single category. Within one such category, however, qualitative differences have been found by a different criterion, namely that of relative specificity for the two natural substrates, under certain conditions.

Whether or not a given *ad-4* allele is capable of reverting to a condition equivalent to wild type in terms of its level of restored enzyme activity appears to depend on the type of damage produced by the previous mutational event or events giving rise to the allele. Four out of five primary *ad-4* alleles yielded some reverse mutants quantitatively and qualitatively equivalent to wild type by the genetic and enzymological tests employed. Since the evidence indicates that at least some

of these *ad-4* mutants were of X-ray origin and that X-rays can produce reverse mutants indistinguishable from wild type, these results support the view that X-rays can induce complete reverse mutation of mutants originally produced by X-rays.

Additional mutational studies were performed with the one allele (F12) which failed to yield any primary revertants equivalent to wild type. These studies involved the production of secondary *ad-4* mutants from such primary *ad-4* revertants by forward mutation, and the subsequent induction of secondary reversions in such secondary mutants. The results indicate that secondary revertants derived from primary revertants which are restricted in that they produce an enzyme having a subnormal amount of activity are in some cases similarly restricted, while in other cases such secondary revertants surpass the primary revertants in the level of restored enzyme activity.

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