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*ENZYME COMPLEMENTATION IN VITRO BETWEEN ADENYLOSUCCINASELESS MUTANTS OF NEUROSPORA CRASSA**

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Previous studies[1] have shown that certain mutant strains of *Neurospora crassa* lacking detectable adenylosuccinase activity, and hence unable to grow in the absence of exogenous adenine, complement one another in certain combinations when grown as heterocaryons. Such complementation results in adenine-independent growth, and certain of the resulting heterocaryons possess a maximum of 25 per cent of wild-type adenylosuccinase activity. The pattern of complementation has been depicted as a complementation map of the *ad-4* locus and suggests a structural correlation between gene and gene product.[2-4] These results led to the hypothesis that complementation involves a cytoplasmic exchange mechanism operating at either the RNA or polypeptide level. The work of Singer and Itano,[5] demonstrating the formation of hybrid protein *in vitro*, lends further support to the suggestion that exchange at the polypeptide level could provide a mechanism for complementation. In addition, such a mechanism could also explain the restoration of enzyme activity in heterocaryons to a maximum level not exceeding 25 per cent of wild type. Other recent studies[6-9] suggest related types of exchange mechanisms that could also yield hybrid protein or protein complexes.

The above hypothesis has received additional support with the demonstration of *in vitro* complementation. By mixing mycelial homogenates from certain pairs of complementing adenylosuccinaseless *Neurospora* mutants grown separately, adenylosuccinase activity as high as 20 per cent of that obtained from *in vivo* complementation has been recovered. The same procedure performed with complementing mutants, singly, or with mixtures of non-complementing mutants, has failed to restore detectable enzyme activity. Earlier attempts[1] to detect enzyme activity in mixtures of extracts from complementing *ad-4* mutants gave negative results.

Materials and Methods.—Data on the origin and other characteristics of the *ad-4* mutants employed in these studies have been presented.[4]

The following general procedure has been found to be the most effective in demonstrating *in vitro* complementation in mixing experiments. Mutant cultures are grown separately for 3–4 days at 25°C on Fries minimal medium containing 100

μg/ml of adenine sulfate. Mycelial pads are removed, washed in chilled distilled water, pressed dry on absorbent paper and quick frozen. Frozen samples are mixed, and the cells are disrupted in a Hughes Press at 5000 psi. The frozen powder from the mixed samples is taken from the press and diluted to a liquid paste condition with $M/20$ tris HCl buffer containing 10^{-3} M reduced glutathione (pH 7.0) and placed on a shaker for 10–15 min or until the frozen extract thaws. The pH is adjusted to approximately 8.0 and the mixture diluted further (usually a two-fold dilution); the mixture is agitated for another hour at 4°C. Cell debris is removed by centrifugation at about 50,000 $\times$ g for 30 min. The supernatant solution is further purified by column chromatography on DEAE cellulose.[10] The column purification is not essential to the recovery of enzyme activity, but some of the ultraviolet-absorbing material that would otherwise interfere with the assay is removed. The assay used is a measure of decrease in absorption at 280 mμ as the reaction proceeds from adenosine monophosphate succinate (AMP-S) to adenosine monophosphate (AMP).[1]

The assumption that the decrease in absorption observed at 280 mμ is a measure of the rate of conversion of AMP-S to AMP is based on spectral scans during the reaction. These scans show a change in maximum absorption from 267 mμ (AMP-S peak) to near 260 mμ (AMP peak). This is the same spectral change observed when the wild-type enzyme is used, but a change that does not occur when single mutant extracts are used. A maximum change in absorption occurs at 280 mμ when either wild-type enzyme or enzyme recovered as a result of *in vitro* or *in vivo* complementation is reacted with the AMP-S substrate. The rate of change in absorption is measured on a recording spectrophotometer and is linear for more than 30 min; thus quantitative determinations can be quite precise.

Results and Discussion.—The results of various mixing experiments performed to date are presented in Table 1. At this stage of the investigation, it appears premature to draw many conclusions about the *in vitro* restoration of enzyme activity except that it is accomplished by the procedure described. Other attempts using variations of this procedure and other extraction procedures have failed to restore enzyme activity. Negative results were obtained whenever mycelia were lyophilized before mixing in the extraction buffer; similarly, when the mycelia were ruptured by grinding in sand with mortar and pestle, no enzyme activity was recovered. From the mixing experiments that have been performed, it appears that the process through which restoration of enzyme activity is accomplished is short-lived under the particular experimental conditions used. Mixing must take place within 5–10 min after the extracts have thawed or no enzyme activity is restored. It does not seem likely that the procedure used exerts its influence through the activation of an inactive enzyme since mutants treated alone exhibit no enzyme activity.

The presence of 10^{-3} M reduced glutathione during extraction appears to improve the recovery of enzyme activity, and there is some indication that ATP enhances the recovery, but highly refined quantitative measurements are not possible at this time.

Additional data have also been obtained from further studies involving *in vivo* restoration of enzyme activity. These data support earlier findings[4] of a correlation between complementation map distance and the levels of adenylosuccinase

TABLE 1

ADENYLOSUCCINASE ACTIVITY RESTORED THROUGH *IN VITRO* COMPLEMENTATION

Mutants	Additives During Extraction	Specific Activity (ΔA/mg. P/min/ml)	% of *in Vivo* Activity
F4 and F39 (heterocaryon)	None (no significant variation with additives present)	1.90	100.0
F4 and F39 (mixture)	None	0.19	10.0
		0.20	10.6
	ATP (Ave. of 3 experiments)	0.30	15.8
	ATP + GSH + NaF	0.31	16.4
		0.28	14.7
F4 and F48 (heterocaryon)	None (no significant variation with additives present)	1.60	100.0
F4 and F48 (mixture)	None	0.15	9.4
	GSH	0.33	20.6
F39 and F31 (heterocaryon)	None	1.17	100.0
F39 and F31 (mixture)	None	0.20	17.1
F4 and F31 (heterocaryon)	None	0.80	100.0
F4 and F31 (mixture)	None	0.04	5.0
F4	All variations	0.00	0.0
F39	All variations	0.00	0.0
F48	All variations	0.00	0.0
F31	All variations	0.00	0.0
F4 and F2* (mixture)	GSH	0.00	0.0
F39 and F2* (mixture)	GSH	0.00	0.0

* Non-complementing combination *in vivo*.

activity found in interallelic heterocaryons. The results plotted in Figure 1 indicate a steady increase of enzyme activity with increasing map distance up to a separation of about 4 cistrons, at which point no further significant increase in activity is observed. According to the hypothesis,[4] if the sites of damage in the two different defective gene products produced by the two complementing mutant nuclei are sufficiently widely separated such that random exchange can occur, then such a heterocaryon should yield 25 per cent of wild-type enzyme activity. This theoretical maximum of 25 per cent was in fact the maximum adenylosuccinase yield observed. However, an attempt to establish the precise nature of this correlation between map distance and enzyme activity does not yet appear justified in view of the limited data and the variability that exists.

Obvious irregularities in the plot of map distance vs. enzyme activity are limited to one or two cases. The one adjacent cistron combination that yields 10 per cent of wild-type enzyme activity may be such an exception. However, this discrepancy may be more apparent than real. For example, it is not yet certain that all of the cistrons have been located at the *ad-4* locus. If a cistron not yet detected exists between the two presumably adjacent cistrons, the 10 per cent value observed would fit into the plot of Figure 1 very well. This particular heterocaryon combination presents an additional complication since one of the mutant components (F23) is leaky to the extent of almost one per cent of wild-type adenylosuccinase activity even though it is unable to grow on minimal medium. The leakiness may contribute to the unexpectedly high 10 per cent recovery. The only other adjacent cistron combination studied (F12 × F14) possessed less than one per cent of wild-type enzyme activity. Because of poor growth in other adjacent cistron combinations, assays for enzyme activity were not possible. Other variables involved in making specific activity determinations are either inherent in the assay procedure or are brought about by unknowns present in the crude extract.

Heterocaryons have also been made between F12 reversions that possess only

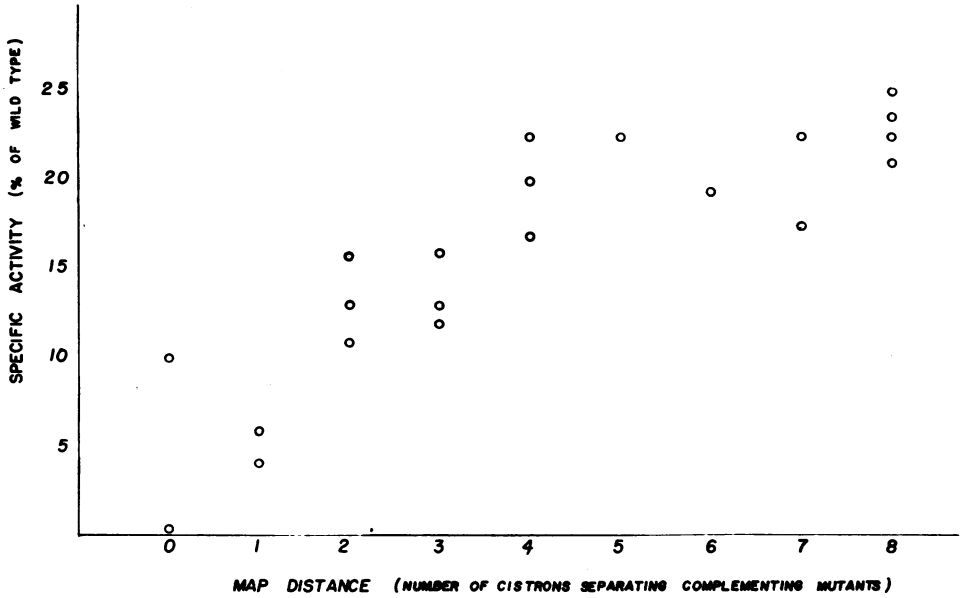

FIG. 1.—Relationship between complementation map distance and adenylosuccinase activity in heterocaryons. Each point plotted represents a heterocaryon composed of different pairwise combinations of adenylosuccinaseless mutants. Many of these figures are averages from several assays of separately grown cultures of the heterocaryons. The specific activity is a measure of the $\Delta A/28$ mg dry wt/20 min/0.77 ml. expressed as a per cent of wild-type activity.

3 per cent of wild-type enzyme activity and mutants that complement F12 and are widely separated from F12 on the complementation map. Since there is now evidence[11] indicating qualitative differences between the enzymes from these reversions and the wild-type enzyme, the assumption can be made that the low level of enzyme activity in the reversions is not due to any quantitative change in the amount of protein synthesized but only to such qualitative changes.[12] On this basis, the hypothesis of random exchange for the wide separation involved predicts a maximum of 25.75 per cent of wild-type enzyme activity. The actual figures obtained from these heterocaryons were 24.3, 24.0, 22.0, and 22.4 per cent of wild-type adenylosuccinase activity.

Until more is understood about *in vitro* complementation, definitive statements concerning the mechanism involved cannot be made. Nevertheless, the present system appears to provide an excellent opportunity for studying this problem.

Summary.—*In vitro* complementation between homogenates from *Neurospora* adenylosuccinaseless mutants resulting in restored adenylosuccinase activity is described. As much as 20 per cent of the activity recovered from *in vivo* complementation in heterocaryons has been restored by the *in vitro* mixing procedure. If the homogenates from two complementing mutants are not mixed immediately after the mycelia have been disrupted, enzyme activity is not recovered. No enzyme activity has been detected when homogenates from non-complementing mutants are mixed. *In vivo* complementation results in the restoration of enzyme activity to a maximum level not exceeding 25 per cent of wild-type. There is a direct correlation between enzyme activity recovered from interallelic heterocaryons and distance between the mutant components on the complementation

map. These results support the hypothesis previously proposed that, in general, interallelic complementation in enzyme formation involves a cytoplasmic exchange mechanism resulting in the formation of hybrid protein.

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LOWER BOUNDS FOR EIGENVALUES WITH APPLICATION TO THE HELIUM ATOM

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In the present paper we shall outline a method for finding lower bounds to the discrete eigenvalues of a self-adjoint operator; upper bounds are always comparatively easy to obtain by the Rayleigh-Ritz technique. Our procedure for lower bounds goes back to the method of A. Weinstein,[1] which introduces an explicitly solvable *base problem* with lower eigenvalues. A. Weinstein links the base problem to the given problem by a sequence of *intermediate problems* which can be solved in terms of the base problem and which improve the lower bounds. In his work on plates these problems are obtained by changing the boundary conditions. By combining his lower bounds with the upper bounds given by the Rayleigh-Ritz method he obtained an accuracy of up to 0.18 per cent.

Later Aronszajn[2] emphasized that a base problem can be obtained by a change of the operator and outlined the construction of the intermediate problems. In this paper we simplify Aronszajn's method by constructing intermediate problems in such a way that in order to solve them in terms of the base problem one need only solve an algebraic equation instead of a transcendental equation. As an applica-