A STRUCTURAL GENE MUTATION IN SALMONELLA TYPHIMURIUM RESULTING IN REPRESSIBILITY OF ADENYLOSUCCINASE*

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Previous work[1] with adenine-requiring auxotrophic mutants of *Salmonella typhimurium* showed that a sequential blockade in the biosynthesis of adenylic acid was due to the genetic loss of the bifunctional enzyme, adenylosuccinase (adenylosuccinate-AMP lyase, EC 4.3.2.2). The phenotypic consequence of this mutation was a specific growth requirement for adenine and the accumulation of the substrate of the first reaction, succinyl-AICAR. By transduction techniques the structural gene for this enzyme was localized on the *Salmonella* chromosome in a region designated as the *pur-B* locus. Subsequently, *in vivo* complementation was demonstrated among all the available *pur-B* mutants although none of these mutants showed any independent adenylosuccinase activity under the usual conditions of assay.[2]

A new class of *pur-B* mutants with a uniquely altered adenylosuccinase has recently been identified. Examination of crude extracts for adenylosuccinase activity showed that this class of *pur-B* mutants contained a specific activity which was approximately $^1/_5$ the adenylosuccinase activity measured in extracts of the wild type. The growth pattern of the members of this group, as well as the variation of their adenylosuccinase activity with nutritional history, suggested an unusual repression effect. Comparisons between the wild-type and mutant adenylosuccinase revealed several differences in stability characteristics. Moreover, the failure to demonstrate any gene product, which was cross-reactive with the enzyme-neutralizing antibody, in extracts prepared from mutant cells grown in excess adenine further suggested a repression concomitant with the mutant alteration. This communication will present the results of studies with *Salmonella typhimurium*, strain *pur-91*, a representative of the new class of *pur-B* mutants.

Materials and Methods.—The organisms employed in these studies were auxotrophic mutants of *Salmonella typhimurium* strain LT_2 obtained from the collection of Dr. M. Demerec. Bacteriophage lysates were prepared from the temperate transducing phage, PL 22 (H_1), and transductions to prototrophy were determined by plating on minimal media. The preparation of phage stocks and their use in transduction was in accordance with methods previously described.[3] Minimal medium consisted of the E medium of Vogel and Bonner[4] plus 0.2% glucose. Where casein hydrolysate medium is indicated, it represents the minimal medium to which casein hydrolysate (Nutritional Biochemicals, vitamin-free) was added to a final concentration of 0.1%. In the growth studies described, purines were added to the minimal medium.

Unless otherwise indicated, extracts were prepared from cells grown in the casein hydrolysate medium. Cells were harvested at 16–18 hr, washed twice with distilled water, and resuspended at 0.5-gm cells (wet weight) per ml in cold 0.03 *M* potassium phosphate buffer (pH 7.2). The chilled suspension was then disrupted by means of a French pressure cell at 6000 psi. The extract was centrifuged in the cold at 11,000 × *g* for 15 min to remove cellular debris, and the supernate was then recentrifuged at 35,000 × *g* for 2 hr.

The method of Gots and Gollub[1] was followed in the preparation of succinyl-AICAR from *E.*

coli B-97. AMPS was prepared from baker's yeast (Fleischmann's type 18-20) by the method of Carter and Cohen.[5]

Adenylosuccinase activity was routinely assayed with the Zeiss spectrophotometer by measuring the rate of decrease in absorbancy at 280 mμ when enzyme (0.001–0.05 units in approximately 0.03 ml) was added to a 3-ml system containing 0.2 μmoles of AMPS in 0.03 *M* potassium phosphate buffer at pH 7.2. A decrease in absorbance of 1.0 per minute was defined as one unit of activity.

Activity with succinyl-AICAR as substrate was measured by incubating for 3 min at pH 7.8, a reaction mixture of 0.4 ml containing 0.03 ml enzyme, 0.45 μM KPO_4 and succinyl-AICAR (0.02–0.12 μM). The reaction was stopped by the addition of 0.15 ml of 30% TCA and the AICAR produced was measured by the Bratton and Marshall[6] test for diazotizable amines as modified by Lukens and Buchanan.[7] The reagents in 0.05-ml amounts were added at 4-min intervals and samples were read in microcells after 5 min by setting the enzyme blank at 100% transmittance and recording the absorbance of the colored derivative at 540 mμ. These readings were corrected for controls where acid was added prior to enzyme.

Neutralization of adenylosuccinase by antibody[8] prepared against partially purified wild-type enzyme was studied by means of the AMPS reaction system described above. In the experiments reported, 0.1 ml of a 1:3 dilution of rabbit antiserum was added to the enzyme in phosphate buffer; substrate was added immediately following mixing and enzyme activity measured as before. The test for enzymatically inactive material which was immunologically cross-reactive (CRM) with the wild-type enzyme was made by repeated adsorptions of the anti-adenylosuccinase sera with cell-free extracts of various mutants. After a period of treatment (generally one week), no additional precipitate could be obtained by centrifugation, and the adsorbed sera were examined for neutralizing ability as described above.

Estimation of the molecular weight of adenylosuccinase was made by sedimentation in a 5–20% sucrose density gradient according to the procedure described by Martin and Ames[9] for molecular weight determinations of protein mixtures. Crude extract (0.1 ml, 2% in protein) was layered on a 4.6-ml gradient. Bovine liver catalase (Sigma; 0.1 ml at 0.4 mg/ml) with a molecular weight of 200,000 was added as a reference and assayed by the methods described in the above paper.

Results and Discussion.—Table 1 summarizes the genetic characterization of the new class of *pur-B* mutants which will be referred to as "leaky" in order to differentiate them from previously described *pur-B* mutants which are designated as "complete." When the "leaky" mutants were employed as donors in transductions with "complete" *pur-B* mutants as recipients, low numbers of wild-type recom-

TABLE 1

TRANSDUCTIONS WITH "LEAKY" *pur-B* MUTANTS AS DONORS

	Donors								
	pur-91			*pur-93*			*pur-96*		
	Recombinants			Recombinants			Recombinants		
Recipients	WT	DT	WT/DT	WT	DT	WT/DT	WT	DT	WT/DT
purB-12	2	524	0.0038	3	776	0.0039	0	360	0
	4	830	0.0048	2	860	0.0023	1	255	0.0039
purB-103	3	340	0.0088	4	377	0.0160	2	>1000	<0.002
	1	156	0.0064	3	180	0.0167	0	73	0
purB-73	2	115	0.0174	2	201	0.0100	3	41	0.0732
	14	550	0.0254	18	530	0.0340	3	190	0.0158
purB-82	8	795	0.0101	2	997	0.0020	0	376	—
purA-155	586	0	—	705	0	—	526	0	—
	469	0	—	764	0	—	447	0	—
purD-10	127	0	—	183	0	—	122	0	—
	131	0	—	136	0	—	112	0	—

Bacteriophage, at a multiplicity of 5, were added to 10^9 bacteria in a 1-ml vol of nutrient broth. Mixtures were incubated for 5 min at 37°C to allow adsorption, and 0.1-ml aliquots were plated on minimal medium and incubated at 37°C.

WT, transductions to prototrophy which appeared within 24–48 hr. DT, minute colonies resembling donor bacteria in colony size and time of appearance on the minimal medium. WT/DT, ratio of large to small colonies scored after a 5-day incubation period. Values for two experiments, each the average of duplicate plates, are shown.

TABLE 2

COMPARISON OF ADENYLOSUCCINASE ACTIVITY OF WILD-TYPE AND *pur-91* MUTANT

Growth medium	Assay*	Adenylosuccinase Activity (units/mg protein) WT	*pur-91*	WT/ *pur-91*
Minimal	A	6.45	1.30	4.96
Minimal + casein hydrolysate	A	6.20	1.25	4.96
	B	7.70	1.68	4.58
Minimal + adenine (20 μg/ml)	A	5.60	1.56	3.60
Minimal + adenine (50 μg/ml)	A	3.08	<0.09	>30
	B	1.64	<0.04	

* Assay A = AMPS → AMP (SA × 10^2); assay B = succinyl-AICAR → AICAR.

binants were observed when plated on a minimal medium. In addition, large numbers of donor-phenotype colonies appeared after approximately 4 days of incubation. This indicates close linkage and apparent allelism. Typical transductions with nonallelic mutant recipients showed the expected high frequency of wild-type recombinants and no recovery of donor phenotype. Since the "leaky" mutants eventually grow on the minimal medium, they could not be used as recipients and therefore it is not known conclusively that they represent mutations at different sites. Comparison of the ratios of wild-type to donor-type colonies obtained for each "leaky" mutant donor with respect to any particular "complete" *pur-B* recipient indicates that the mutational sites resulting in "leakiness" of this type are not identical and are probably clustered. The ratio of wild-type to donor-type colonies for the "leaky" mutant donors with respect to each of the "complete" *pur-B* recipients suggests that the location of these "leaky" mutations may be toward one end of the *pur-B* locus.

Although mutants of this group accumulate the succinyl-AICAR intermediate, they differ from the "complete" *pur-B* mutants with respect to growth requirements. When stationary cells were transferred from adenine-containing media to a minimal medium, these "leaky" mutants grew after a lag of 12–42 hr. This lag was not demonstrable when the culture which had finally reached maximum stationary phase in minimal medium was transferred again to minimal medium where the mutant subsequently grew with a generation time which was approximately 2× longer than that of the wild type (Fig. 1) and contained approximately $^1/_5$ of the wild-type adenylosuccinase specific activity (Table 2). It was found that

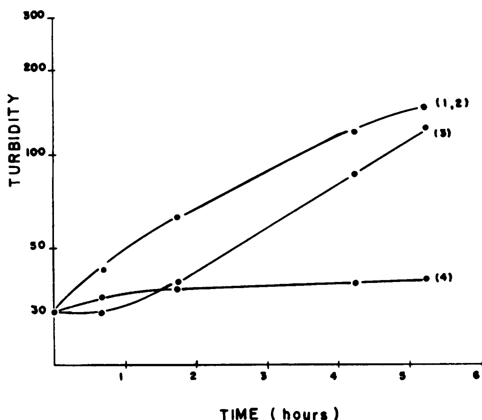

FIG. 1.—Growth pattern of *pur-91;* influence of nutritional history. Starter cultures were harvested at maximum stationary, washed twice with 0.03 *M* KPO_4 buffer pH 7.2, centrifuged, and resuspended in the appropriate medium at approximately 2 × 10^7 cells/ml. Numbers on curves refer to order of transfer into unsupplemented minimal media or minimal media containing adenine (20 μg/ml) as follows: (*1*) minimal to adenine, (*2*) minimal to minimal, (*3*) adenine to adenine, (*4*) adenine to minimal. The generation time for curves (*1*) and (*2*) is 120 min as compared with 68 min for similar treatment of wild type.

addition of casein hydrolysate to a final concentration of 0.1 per cent eliminated the growth lag of these mutants in minimal medium while the relationship of the mutant's generation time to that of the wild type remained the same.[10] When extracts were prepared from these mutants grown in the minimal casein hydrolysate medium, approximately $^1/_5$ of the wild-type specific activity was observed here also. Table 2 compares adenylosuccinase activity in crude extracts from wild type with that from *pur-91*, a representative "leaky" mutant. The activity of the mutant extracts was consistently $^1/_5$ that of the wild type. Mixtures of the wild-type and mutant extracts did not indicate the presence of an inhibitor. Neither extensive dialysis nor precipitation of nucleic acids with streptomycin altered the ratio of activities. Moreover, the ratio of mutant to wild-type activity showed no appreciable change on purification.[11] Adenylosuccinase activity in the wild-type strain varies with the nutritional history of the cells to a limited extent. However, although the wild-type activity could be repressed to $^1/_2$ the usual value by growing the cells in minimal medium supplemented with adenine (50 μg/ml), the mutant enzymic activity was completely eliminated under these conditions (Table 2).

When the crude extracts were also examined for gross differences with respect to enzyme kinetics, the K_m for AMPS for both the wild-type and mutant adenylosuccinase was shown to be 1.4×10^{-5} *M*. Both activities showed the same pH dependency in phosphate buffers.[11] Since the genetic lesion evidently had not resulted in extensive alteration of the substrate site, studies were directed toward detecting a change in some physical property which might indicate a conformational basis for the repression effect shown in Table 2. Figure 2 shows the results obtained when mutant and wild-type enzyme were preincubated at 60°C for various intervals preceding assay. The marked heat lability of the mutant adenylosuccinase is readily apparent, since complete loss of activity occurred within 10 min while the wild-type activity was only slightly decreased after 40 min at 60°C.

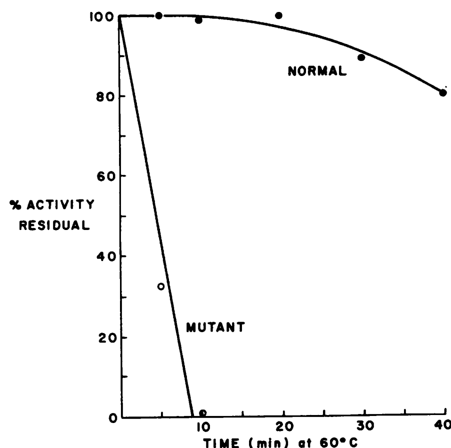

FIG. 2.—Heat inactivation of adenylosuccinase in crude extracts (15 mg protein/ml). At each interval, samples were diluted 1:10 in ice-cold KPO_4 buffer (0.03 *M*, pH 7.2) and kept in ice bath until assayed.

The activity of adenylosuccinase is markedly dependent upon salt concentration, and Figure 3 (*bottom*) shows the effect of phosphate concentration on the activity of mutant and wild-type enzyme. Although a slight shift in maximum was observed for the mutant, changes in concentration of phosphate affected both activities similarly. However, that the activity-concentration relationship was not entirely due to ionic strength is also indicated in Figure 3 (*top*) which shows the results of varying KCl concentrations over the same ionic strength range, buffered at the same pH. In the case of the mutant enzyme, the inhibition by KCl was quite pronounced, and complete loss of activity could be demonstrated at a concentration where the wild type still retained 50 per cent of its original activity.

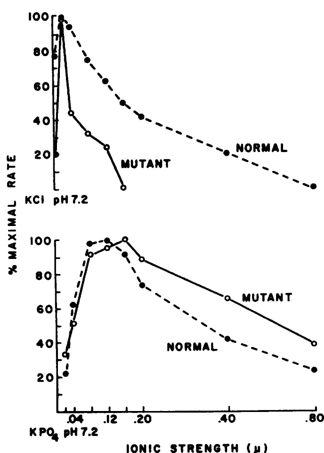

FIG. 3.—Dependence of adenylosuccinase activity upon ionic strength; comparison of chloride and phosphate ionic effect. Crude extracts (15 mg/ml protein) were diluted 1:10 in the appropriate buffer and assayed directly by addition of AMPS. Results are reported as % maximal rate obtained for each system. Maximum rate with KCl = 50% of PO_4 rate for wild type; 30% of PO_4 rate for mutant.

The relative effect of chloride and phosphate on adenylosuccinase activity probably reflects the generality of the effects of certain salts on the stability of macromolecular conformations.[12] Therefore, the effect of Cl^- on the mutant enzyme is most likely significant in terms of the disruptive effect of Cl^- enhanced by the relatively unstable character of the native conformation of mutant enzyme as compared to wild-type enzyme.

The above results implied: (1) the mutant produced a quantity of altered enzyme which was repressed to $^1/_5$ the wild-type amount of adenylosuccinase, or (2) the altered enzyme produced by the mutant was only $^1/_5$ as active as the wild-type enzyme with respect to its turnover number. Consequently, immunologic studies were pursued to detect the amount of enzyme antigen present in crude extracts prepared from the normal and mutant organisms.

Neutralization was immediate and the dissociation constant for the enzyme-antibody complex apparently was extremely small. Therefore, antienzyme antibody could be treated as an essentially irreversible (or pseudoirreversible) inhibitor demonstrating a dependence on enzyme concentration.[13] If the concentration of enzyme were varied in a system containing a constant amount of antibody, a linear relationship for activity versus enzyme concentration should have resulted, having a slope parallel to the control and intersecting the abscissa at the point of complete enzyme neutralization.

Figure 4 shows the results of such a system with adenylosuccinase. The abscissa represents units of enzyme added on the basis of activity in the presence of control serum, and the ordinate represents units of activity measurable in the presence of a constant amount of antiserum. If the straight lines obtained for both the mutant and the wild-type activities are extrapolated to the abscissa, they intercept at the same point which correlates the enzymic activity for each organism with equivalent amounts of antigenic material. If the difference in specific activity between the mutant and wild-type extracts were due to a less active enzyme in the case of the mutant, a greater number of antibody molecules would have been required to neutralize a unit of mutant enzymic activity as compared to a unit of wild-type enzymic activity. Since, as shown in Figure 4, the same antibody concentration neutralized an equal number of mutant and wild-type enzyme units, it appears that the second possibility mentioned above may be excluded and that the *pur-91* adenylosuccinase is quantitatively deficient as well as qualitatively defective (Figs. 2 and 3). The hypothesis that this decrease in amount of enzyme produced by *pur-91* is probably a consequence of the mutational event that resulted in an altered polypeptide chain will be discussed below.

Additional information was obtained from the experiment shown in Figure 4. The results also excluded the presence of any inactive forms of adenylosuccinase

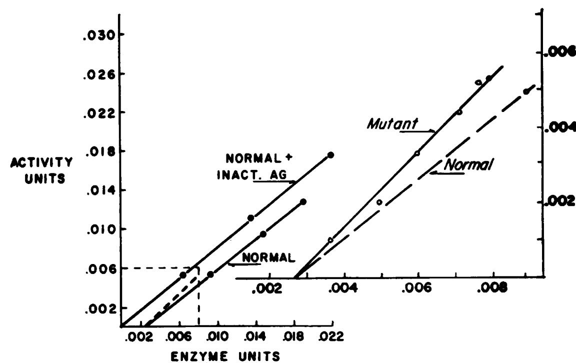

FIG. 4.—Neutralization of adenylosuccinase activity by rabbit antiserum prepared against partially purified wild-type extract. Aliquots of normal and mutant crude extracts (15 mg protein/ml) were added to 0.03 *M* KPO_4 pH 7.2 buffer containing 0.1 ml of a 1:3 dilution of serum. AMPS was added and reactions were assayed immediately following mixing. The abscissa refers to the number of enzyme units added to the system, and the ordinate refers to the activity measured after antibody action. (Deviation from ideality for the slope of normal enzyme was subsequently found to be a nonspecific serum effect.) The coordinates shown on right represent the plot of normal and mutant activities on a $^1/_4$ scale in order to amplify the intercept shown in the enclosed area in the lower left quadrant. "Normal" activity of wild-type enzyme in the presence of unadsorbed serum or serum preadsorbed with an extract prepared from *ade-91* repressed (see *Materials and Methods*). "*Normal + Inact. Ag*" = activity of wild-type enzyme in the presence of several sera, each preadsorbed with crude extracts prepared from cells of *pur-103, −73, −82, −12,* respectively, following growth in (*a*) minimal adenine, 20 μg/ml, or (*b*) repressor adenine, 50 μg/ml, medium.

in the case of the mutant since their presence would have shifted the complete neutralization point toward the origin.[14] Such a shift was observed when the antiserum used in this experiment was previously adsorbed with an enzymically inactive extract from a "complete" *pur-B* mutant (see *Materials and Methods*). By this method, CRM has been demonstrated in extracts of all the "complete" *pur-B* mutants tested (see legend, Fig. 4; "Normal + Inact. Ag."). However, when the antiserum was previously adsorbed with the enzymically inactive extract prepared from the "leaky" mutant grown under conditions where repression of enzyme activity occurred (Table 2), no inactive antigen was detectable by this method. Moreover, no effect on the presence of CRM in extracts of the "complete" *pur-B* mutants could be shown following growth of these mutants in excess adenine where the *pur-91* adenylosuccinase was repressed.

Figure 5 shows that the molecular weight for the *Salmonella* adenylosuccinase is approximately 190,000 and that this value is comparable for both the wild-type and mutant enzymes. It is interesting that a similar value has been obtained for the molecular weight of adenylosuccinase in *Neurospora crassa*.[15] Recently, physical studies on the purified *Neurospora* enzyme have demonstrated the subunit nature of the enzyme molecule which had been inferred from *in vivo* and *in vitro* complementation in that system.[15–17] Although the effect of the *pur-91* mutation upon the quantitative and qualitative aspects of adenylosuccinase messenger production cannot be ignored, an explanation of the growth, repression, enzymic, and immunologic data obtained for this organism might best be sought at the level of the complexity of the quarternary structure of the enzyme. Since the mutant produces $^1/_5$ the wild-type enzyme, and since the consequence of the structural gene mutation

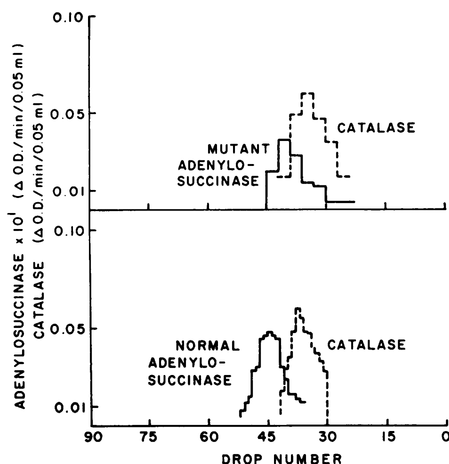

FIG. 5.—Distribution of adenylosuccinase activity following sedimentation in a 5–20% sucrose density gradient at 37,000 rpm for 8 hr. Due to the low level of activity, in the mutant case, the limit of resolution of enzyme activity is 3 drops.

appears to be a less stable polymer, the amount of mutant enzyme formed may be proportional to the degree of interaction between defective subunits. However, two basically different models are possible here.

According to one hypothesis, the amount of mutant enzyme formed would be proportional to the degree of interaction between defective subunits which are produced in normal amounts, most remaining "uncoupled" in a cytoplasmic milieu. The condition for repression here would be that adenine, or a derivative, when present in high concentrations, interferes in some way with this low level of polymerization. However, another model for interaction between defective subunits, which would also affect the number of subunits synthesized, is equally consistent with these observations. It is possible that message for the monomer might be released for synthesis of new monomer only after combination of its product with another completed, or partially synthesized, subunit. The net effect of this latter hypothesis would be less total monomers in the case of mutant *pur-91*, since the production of subunits of mutant adenylosuccinase would be limited by the degree of interaction between them.

Operationally, one should be able to differentiate between these two models by some system which would detect uncoupled subunits in extracts of *pur-91*. As discussed in connection with the results shown in Figure 4, these subunits would be inactive with respect to antiadenylosuccinase antibody. Therefore, the experimental approach has been based on the possibility of inducing polymerization of these subunits so that they may be detected enzymically or immunologically. Preliminary trials where enzyme activity is measured following incubation at various temperatures and ionic strengths have shown the formation of a measurable amount of adenylosuccinase activity in *pur-91* repressed extracts following extensive dialysis.[18] However, it is impossible to assess from these early results whether the normal amount of intact enzyme can be quantitatively accounted for as subunits in the mutant case. Such an audit would have to be made if the mutational effect on repressibility of adenylosuccinase were to be ascribed solely to the level of interactions between preformed polypeptides in a cytoplasmic milieu.

Summary.—The data discussed above indicate that a mutation, *pur-91*, in the structural gene for adenylosuccinase has resulted in: (1) an adenylosuccinase altered with respect to stability characteristics, (2) a deficiency in the amount of specific enzyme synthesized by the mutant, and (3) acute repressibility of adenylosuccinase.

Abbreviations used: AICAR, 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide; succinyl-

AICAR, 5'-phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole; AMP, adenosine-5'-phosphate; AMPS, adenylosuccinic acid; CRM, immunologically cross-reactive material.

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*PHOTOREACTIVATION OF TOBACCO MOSAIC VIRUS RIBONUCLEIC ACID FOLLOWING NEAR ULTRAVIOLET IRRADIATION**

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Extensive investigations of the repair of UV-damaged DNA have led to the elucidation of a number of mechanisms, including photoreactivation, dark repair, liquid-holding repair, UV reactivation, and T4 *v* gene repair.[1] A photoreactivating enzyme which cleaves thymine dimers in UV-damaged DNA has been purified (for review, see ref. 2), and an enzyme system for dark repair which excises thymine dimers and may be involved in the subsequent reformation of the intact double helix has also been implicated.[3–6]

The nature of the lesions in UV-damaged RNA and the repair mechanisms for this nucleic acid are not clear. Attempts to demonstrate photoreactivation of M12, an RNA-containing bacteriophage, proved fruitless.[7] No reports of repair of UV-damaged RNA in bacteria have appeared, but a number of intact plant viruses, all of which contain RNA, do photoreactivate.[8] Although neither TMV nor RNA extracted from the irradiated virus will photoreactivate, free TMV-RNA, irradiated