## Genetic Alteration of Adenylic Pyrophosphorylase in Salmonella

Abstract. *A mutant of* Salmonella typhimurium, *resistant to inhibition by 2, 6-diaminopurine, differs from its sensitive wild-type parent by an altered adenylic acid pyrophosphorylase. The altered enzyme, though still active with adenine as substrate, is inactive in the conversion of diaminopurine to its nucleotide. It also differs from the wild-type enzyme in a number of physical and chemical properties.*

The purine nucleotide pyrophosphorylases convert purine bases to their respective ribonucleotides by pyrophosphorolytic condensation with 5-phosphoribosyl-1-pyrophosphate (PRPP). At least three distinct pyrophosphorylases are known which can convert the naturally occurring purine bases; one for adenine, one for guanine or hypoxanthine, and one for xanthine (*1, 2*). These enzymes also act by converting synthetic purine analogs to their respective ribonucleotides and, in most cases, this action is a prerequisite for providing an inhibitory form of the analog. When these enzymes are altered by mutational events, the analog cannot be converted to their inhibitory forms and a mutant results which can grow in the presence of the analog. Selection for analog-resistant mutants thus provides a convenient way for isolating organisms with genetically altered pyrophosphorylases. A variety of such mutants have been isolated from several bacterial species (*3, 4*). This report deals with the analysis of one such case, the alteration of adenylic pyrophosphorylase (AMP-PPase) in a mutant of *Salmonella typhimurium* resistant to 2, 6-diaminopurine.

We have previously reported (*5*) on the isolation and description of two distinct mutants, differing in phenotype, which are resistant to the inhibitory action of diaminopurine. Both apparently owe their resistance to the inability to convert diaminopurine to its ribonucleotide. One (strain *dap-r-3*) shows a number of pleiotropic metabolic disturbances and is completely devoid of adenylic pyrophosphorylase activity; neither diaminopurine nor adenine can be pyrophosphorylated. The other type mutant, strain *dap-r-6*, shows no associated metabolic alterations and though it is deficient in its ability to pyrophosphorylate diaminopurine, its ability to convert adenine remains unchanged. These two types of mutants thus represent two types of mutational consequences, one which results in loss of enzyme and the other in a modification detected by its inability to pyrophosphorylate the analog. The unhampered enzymic activity towards adenine provides a convenient tool for assay, isolation, and analysis of the altered enzyme.

Methods for culturing the bacteria and preparation of extracts were as previously reported (*4*). The bacteria were harvested after growth in a medium of minimal salts and glucose, and disrupted by passing through a French pressure cell or by grinding with alumina. Nucleic acids were removed by precipitation with $MnCl_2$; the enzymes were isolated by precipitation, at *p*H 5.0 with potassium acetate, dialysis, and application to a diethylaminoethyl-(DEAE)-cellulose column. The enzymes were eluted by a linear NaCl gradient solution ranging from 0 to 0.5*M* in tris-succinate-magnesium-acetate buffer at *p*H 7.6 [tris, 0.01*M*; succinate, 0.004*M*; Mg $(CH_3COO)_2$ 0.005*M*]. A total volume of 400 ml was passed through the column and collected automatically in 3 ml fractions. The fractions were assayed for activity with adenine, 2, 6-diaminopurine, and hypoxanthine. The hypoxanthine served to place inosine monophosphate (IMP)-pyrophosphorylase, a distinctly different enzyme, as an internal reference point for comparison. Assay of pyrophosphorylase activity was based on the measurement of purine-dependent disappearance of ribose after removal of formed nucleotides by adsorption to charcoal (*4*). A unit of enzyme is that amount which can convert 0.02 μmole of PRPP to nucleotide in 1 hour.

It is evident from the results shown in Fig. 1 that the adenylic pyrophosphorylase activity of the mutant gives a different elution pattern from that of the wild type. The mutant enzyme was eluted from the column at a salt concentration two-fifths of that required for elution of the wild-type enzyme. Activity with diaminopurine was found only in fractions from the wild type preparation and only in those that also showed activity with adenine. When the two preparations were mixed in equal parts, each containing the same amount of protein, and applied to the column, the elution pattern showed a resolution of adenylic pyrophosphorylase activity into two distinct peaks. The second peak was that of the wild-type enzyme since enzymatic activity with diaminopurine as substrate occurred only with the fractions in this area. Measurements of the areas under the curves in Fig. 1 showed that the adenylic pyrophosphorylase activity of the mixture was 93.5 percent of the total of the two separate preparations. The IMP-PPase activity of the mixture showed no significant alteration of elution pattern and represented 91.6 percent of the total.

Table 1. Substrate activity (micromoles per hour per milligram of protein) of adenylic acid pyrophosphorylase from wild type, strain LT-2, and from diaminopurine-resistant mutant, strain *dap-r-6*.

| Substrate | Strain | |
|---|---|---|
| | LT-2 | dap-r-6 |
| Adenine | 5.0 | 6.7 |
| 2,6-Diaminopurine | 3.2 | 0.0 |
| 8-Azaadenine | 2.6 | 0.7 |

The adenylic pyrophosphorylase activity recovered from the columns represented about a 40-fold purification over that in the crude extracts. The preparations so obtained from wild-type and mutant were examined for similarities and differences.

From Lineweaver-Burke graphs, the $K_m$ (Michaelis-Menten constant) value

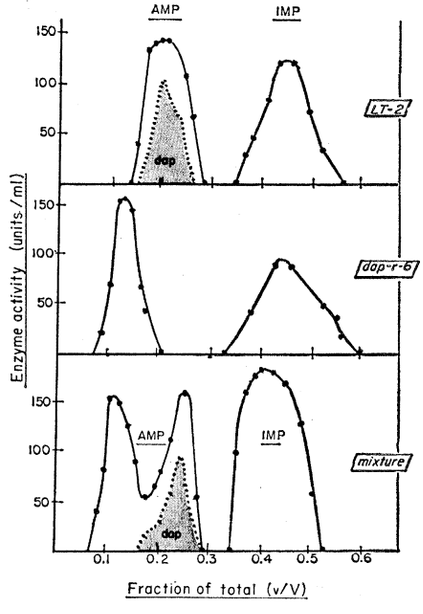


Fig. 1. Elution patterns of pyrophosphorylases from DEAE-cellulose column. The AMP-PPase activity was eluted first and IMP-PPase activity late. Shaded areas under AMP curves refer to activity with diaminopurine (dap). Wild type (Lt-2), at top; mutant (*dap-r-6*), in middle; equal mixture of wild type and mutant at bottom.

with adenine as substrate was $1.0 \times 10^{-5}$ for the wild type, and $0.8 \times 10^{-5}$ for the mutant; and with PRPP as the substrate, the $K_m$ was $3.4 \times 10^{-4}$ for the wild type and $2.6 \times 10^{-4}$ for the mutant. Both enzymes were equally sensitive to inhibition by inorganic pyrophosphate, 60 to 65 percent at $5 \times 10^{-3}M$ and 100 percent at $5 \times 10^{-2}M$, but with $5 \times 10^{-2}M$ sodium fluoride the mutant was only 44 percent inhibited as compared to 100 percent for the wild type. Heat-inactivation showed a greater temperature sensitivity for the mutant enzyme. At 55°C the extent of inactivation was twice that of the wild type. The time required for 50-percent inhibition was 48 seconds for the mutant enzyme compared to 96 seconds for the wild type. This twofold difference in the rates of inactivation of the two enzymes was consistent within the range studies 50° to 60°C and was not significantly altered by either a twofold increase or decrease of enzyme protein concentration. The *p*H for optimum activity was less for the mutant enzyme, 6.8 as compared with 7.6 for the wild-type enzyme. The effect of *p*H was more striking at *p*H 8.0 where wild-type activity remained near optimum but mutant activity was decreased to 47 percent of its optimum. As shown in Table 1, there is no difference in substrate activities, of the two enzymes with adenine as substrate compared to the complete loss of activity in the mutant when diaminopurine is the substrate. Another analog, 8-azaadenine, can be pyrophosphorylated by adenylic pyrophosphorylase and though the mutant retains this activity it is only 27 percent as active as the wild type. No activity for either preparation could be shown with 2-aminopurine, isoguanine (2-oxyadenine), hypoxanthine, guanine, xanthine, 6-mercaptopurine, or 8-azaguanine.

Thus, a new protein species has been created by the genetic alteration which gave rise to the mutant. It is also of interest to note, in terms of active sites, that what represents a mutant form in Salmonella may be the normal form in other organisms, for the adenylic pyrophosphorylase of yeast has been reported to be inactive in pyrophosphorylation with diaminopurine (*1, 6*).

GURUDUTT P. KALLE*
JOSEPH S. GOTS
*Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia 4*

8 NOVEMBER 1963

**References and Notes**

1. A. Kornberg, I. Lieberman, E. S. Simms, *J. Biol. Chem.* **215**, 417 (1955).
2. J. G. Flaks, M. J. Erwin, J. M. Buchanan, *ibid.* **228**, 201 (1957); L. N. Lukens and K. A. Herrington, *Biochim. Biophys. Acta* **24**, 432 (1957); C. E. Carter, *Biochem. Pharmacol.* **2**, 105 (1959); G. P. Kalle and J. S. Gots, *Federation Proc.* **20**, 358 (1961).
3. R. W. Brockman, C. S. Debavadi, P. Stutts, D. J. Hutchinson, *J. Biol. Chem.* **236**, 1471 (1961).
4. G. P. Kalle and J. S. Gots, *Biochim. Biophys. Acta* **53**, 156 (1961).
5. ———, *ibid.* **51**, 130 (1961); *Proc. Soc. Exptl. Biol. Med.* **109**, 277 (1962).
6. Supported by grants from the National Institutes of Health (CA-02790) and the National Science Foundation (G-5496).
* Present address: Biology Division, Atomic Energy Establishment, Trombay, Richardson, and Cruddas Building, Byculla, Bombay 8, India.

26 August 1963

## Fluorescence and Absorption Changes in Chlorella Exposed to Strong Light: The Red Band

Abstract. *The reversible decrease in the apparent intensity of the main red absorption band of chlorophyll* a *at 680 mμ upon strong illumination of* Chlorella *cells, described by Coleman and Rabinowitch in 1957, is shown, by a polarization method, to be due to an increase in the fluorescence yield. True absorption changes are observed at 657 mμ (positive) and 648 mμ (negative).*

The steady-state difference spectrum of *Chlorella* (that is, the absorption of illuminated cells less the absorption of non-illuminated cells), measured in the red region by Coleman and Rabinowitch (*1, 2*) appears complex, with negative bands at 630, 650, 680, and 710 mμ appearing upon irradiation of a cell suspension with white light of high intensity. The difference spectrum of *Chlorella* measured by Kok (*3*) before and after flashes, also shows a complex structure in the red region, with negative bands at 650, 680, and 700 mμ. The prominent band at 680 mμ, noted in both papers, suggested reversible bleaching of chlorophyll *a* during photosynthesis (*2*).

When using a flow system, Strehler and Lynch (*4*) observed no absorption changes at 680 mμ upon irradiating *Chlorella* cell suspensions with light of low intensity. Strehler (*5*) suspected that the apparent absorption changes at 680 mμ, noted by others, were in fact changes in the yield of fluorescence due to the detecting beam, caused by actinic light. Fluorescence due to the actinic beam itself does not affect the measurements, because this light is constant; only the modulated light of the measuring beam, and the modulated fluorescence produced by it, cause a response in the detecting system.

The fluorescence yield of chlorophyll in *Chlorella* is known to increase by a factor of about 2 with increasing intensity of exciting light, when photosynthesis reaches saturation (*6*). The increase in fluorescence yield can thus appear as a negative change in absorption in the difference spectrum. This effect is proportional to the difference between the slopes of the curve "fluorescence intensity as a function of excitation intensity," in low light (measuring beam alone) and high light (measuring beam plus actinic beam). This slope has been shown by Brugger (*7*) to be constant at low intensities of exciting light, but increasing in steepness in the region where photosynthesis becomes light-saturated and approaching another constant value at high light intensity. This can explain the sigmoid shape of the light intensity dependence of the spectral change at 680 mμ, which was observed by Coleman and Rabinowitch (*1, 2*). Coleman (*8*) checked this hypothesis by rerouting his detecting beam so that it traversed the suspension at a right angle to its usual straight path, but concluded that changes in fluorescence due to the detecting beam contributed less than 7 percent to the changes measured at 680 mμ.

The more recent results presented here, however, suggest that an increase in the yield of fluorescence might have produced most, if not all, of the "negative absorption band" observed by Coleman at 680 mμ.

To measure the difference spectrum, we used an instrument developed in our laboratory from Duysens' original instrument (*9*), and similar in principle to that used in the study by Coleman and Rabinowitch. The monochromatic measuring beam (band width, 3.3 mμ), modulated by a rotating disk, was obtained from a 6-volt ribbon filament lamp and passed through a Bausch and Lomb 250-mm focal length grating monochromator, with the entrance and exit slits set at 0.50 mm. The cell suspension of *Chlorella pyrenoidosa* was irradiated at right angles to the detecting beam by blue light from another 6-volt ribbon filament lamp (filtered through Corning color filter No. 4-72). Red filters were placed in front of the detector (photomultiplier RCA 6217) to prevent the actinic light