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Ability of Nonenzymic Nitration or Acetylation of *E. coli* Glutamine Synthetase to Produce Effects Analogous to Enzymic Adenylylation

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Abstract. Treatment of unadenylylated glutamine synthetase from *Escherichia coli* with tetranitromethane or with *N*-acetylimidazole produces alterations in catalytic parameters that are similar to alterations caused by the physiologically important process of adenylylation. All three modification reactions lead to a change in divalent ion requirement for biosynthetic activity; the unmodified enzyme requires Mg^{2+} for activity, whereas the modified enzymes exhibit increased activity with Mn^{2+}. The γ-glutamyl transferase activity of the modified enzyme is more sensitive to feedback inhibition by tryptophan, histidine, CTP, and AMP, and to inhibition by Mg^{2+} or to inactivation by 5 *M* urea. Finally, the pH optimum for the unmodified enzyme is 7.9, while the modified enzymes are more active at pH 6.8.

Since treatment of the enzyme with *N*-acetylimidazole results in a decrease in absorbancy at 278 mμ and treatment with tetranitromethane causes an increase in absorbancy at 428 mμ, the effects of these reagents are probably due to modification of certain tyrosyl groups on the enzyme. However, other evidence indicates that the tyrosyl residues which are susceptible to adenylylation in the adenylyltransferase-catalyzed reaction are not involved in the acetylation or nitration reactions.

The activity of glutamine synthetase in *Escherichia coli* is regulated by repression,[1] cumulative feedback inhibition,[2] and by enzyme-catalyzed adenylylation and deadenylylation of specific tyrosyl groups on the enzyme.[3–6] Adenylylation of the enzyme is accompanied by changes in divalent ion specificity, in the pH optimum, and in the susceptibility to inhibition by various products of glutamine metabolism, and by other effectors.[4,7] Since tetranitromethane and *N*-acetylimidazole are known to react preferentially with tyrosyl groups on protein,[8–10] the effects of these reagents on the catalytic activity of glutamine synthetase was investigated. The data indicate that both reagents modify tyrosyl groups on glutamine synthetase and that this is accompanied by changes in various catalytic parameters that are similar to the changes caused by enzyme catalyzed adenylylation of the enzyme.

Materials and Methods. Chemicals: Uniformly labeled ^{14}C-ATP was obtained from New England Nuclear, Boston, Mass. All amino acids and nucleotides used were purchased from Calbiochem, Los Angeles, Calif. Tetranitromethane was from

Aldrich, Cedar Knolls, N.J., and *N*-acetylimidazole was either prepared by the method of Reddy *et al.*[11] or purchased from K and K, Plainview, N.Y. The purity of the acetylimidazole was ascertained by ultraviolet spectral analysis and by melting-point determination. Snake venom phosphodiesterase (*Crotalus terr. terr.*) was obtained from Boehringer Mannheim, New York, N.Y. All chemicals used were of the highest quality available from commercial sources.

Enzymes: Glutamine synthetase was purified from *E. coli* W by the procedure of Woolfolk *et al.*[1] The preparation containing an average of 7.9 adenylyl groups/mole (designated $E\overline{7.9}$)[12] was isolated from cells grown as previously described.[13] The $E\overline{0.8}$ preparation was from cells grown on a modified medium initially containing 0.11 *M* NH_4Cl and 0.67 *M* glycerol and were harvested 4 hr after the onset of stationary growth induced by NH_4Cl depletion. A large batch of cells grown under these new conditions was kindly provided by Drs. E. T. Phares and N. V. Long, Biology Division, Oak Ridge National Laboratory. The $E\overline{0.95}$ enzyme was obtained by deadenylylating $E\overline{7.9}$ with snake venom phosphodiesterase as described previously.[3] The $E\overline{12}$ preparation was prepared by adenylylation of $E\overline{7.9}$ with ATP in the presence of Mg^{2+}, glutamine, and adenylyltransferase, as previously described.[14,15] The partially purified adenylyltransferase was a generous gift of Dr. S. B. Hennig.

Methods: Glutamine synthetase biosynthetic activity was determined by the method of Woolfolk *et al.*[1] with the exception that the Mg^{2+} and Mn^{2+} assays were at pH 7.5 and 7.2, respectively.[16] The standard conditions of the γ-glutamyl transfer reaction were used,[1] but at pH 7.15.[16] For some experiments a "mixed imidazole buffer assay system" was used in which the standard transfer assay mixture was modified[7,16] to contain 50 mM each of imidazole, 2-methylimidazole, and 2,4-dimethylimidazole buffers (pH 7.15), and 20 mM hydroxylamine, 20 mM glutamine, and 0.3 mM $MnCl_2$. In other experiments, a "saturating assay system" was used which contained 150 mM glutamine, 25 mM hydroxylamine, 20 mM arsenic acid (neutralized to pH 7.0 with triethanolamine), 0.4 mM ADP, 50 mM triethanolamine-dimethylglutarate, 300 mM KCl, and 0.4 mM $MnCl_2$, pH 7.5. The extent of adenylylation of various glutamine synthetase preparations was determined with the mixed imidazole assay system by measuring the transfer activity in the presence and absence of 60 mM $MgCl_2$[7] or by employing the spectrophotometric procedures outlined by Kingdon *et al.*[4]

Adenylylation of nitrated or acetylated $E\overline{0.8}$ glutamine synthetase was achieved by incubating them with ^{14}C-ATP and adenylyltransferase and glutamine.[14,17] The protein-bound ^{14}C-AMP was determined by measuring the amount of radioactivity that was insoluble in boiling trichloroacetic acid.[14,17] Nitration of glutamine synthetase was achieved by adding tetranitromethane, diluted in ethanol, to an incubation mixture containing enzyme, 50 mM 2-methylimidazole, 1 mM $MgCl_2$, and 0.1 *M* KCl at pH 8.1. After 60 min at room temperature, the reaction was terminated by a 25- to 50-fold dilution with the same buffer containing no KCl, which was cooled to 4°C, and aliquots were then assayed directly for enzymatic activity. Sometimes the nitrated enzyme was isolated from the nitration mixture by passage through Sephadex G-25 equilibrated with 50 mM 2-methylimidazole, 1 mM $MgCl_2$, 1 *M* KCl, pH 8.1, and the 3-nitrotyrosine content was estimated from absorbancy measurements at 428 mμ.[8] Acetylation of glutamine synthetase was achieved by adding *N*-acetylimidazole (freshly dissolved in 50 mM 2-methylimidazole, pH 7.6) to a solution of the enzyme in 10 mM imidazole-1 mM $MgCl_2$, pH 7.1. After 60 min at room temperature the acetylation reaction was stopped by diluting the incubation mixture 25- to 50-fold with 10 mM imidazole-1 mM $MgCl_2$, pH 7.1, and cooling to 4°C. For spectral studies the acetylated enzyme was exhaustively dialyzed against 10 mM imidazole-1 mM $MgCl_2$, pH 7.1. Acetylation of the tyrosine hydroxyl group produces a characteristic decrease in absorbancy at 278 mμ.[9]

Protein concentrations of the purified glutamine synthetase preparations were calculated from absorbancy measurements at 290 mμ.[14] A micromodification of the biuret reaction was used to determine the protein concentration of the nitrated and acetylated enzymes.[18]

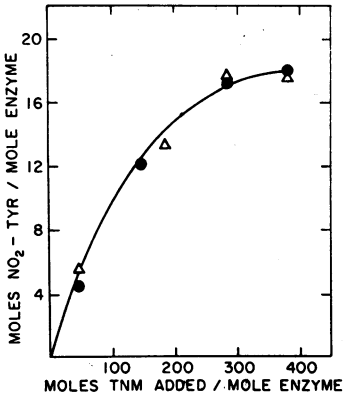

FIG. 1.—Effect of tetranitromethane (TNM) concentration on the nitration of tyrosyl groups of adenylylated and unadenylylated glutamine synthetase. The reaction mixtures contained 20 mg of $E_{\overline{0.95}}$ (●—●) or $E_{\overline{12}}$ (△—△)/ml, and tetranitromethane as indicated. Other conditions and estimation of the nitrotyrosine content from absorbancy measurements at 428 mμ are described in *Materials and Methods*.

Results. Figure 1 shows that under the conditions used (see *Materials and Methods*), the number of tyrosyl residues nitrated increases as the molar ratio of tetranitromethane to glutamine synthetase is increased. Nitration of both unadenylylated and fully adenylylated enzyme is a nearly linear function of the tetranitromethane concentration up to a molar ratio of about 150, at which point about 12 tyrosyl groups (1/subunit of enzyme) are nitrated. Further nitration up to a limiting value of about 18 tyrosyl groups/mole is obtained as the molar excess of tetranitromethane is increased to 350. Since the enzyme contains 192 tyrosyl groups/mole, it is evident that only about 10% of them are nitrated under the conditions used.

Effect of nitration on the catalytic activity of adenylylated and unadenylylated enzyme: Prior incubation of unadenylylated glutamine synthetase with tetranitromethane results in significant alteration in its catalytic activity. Figure 2

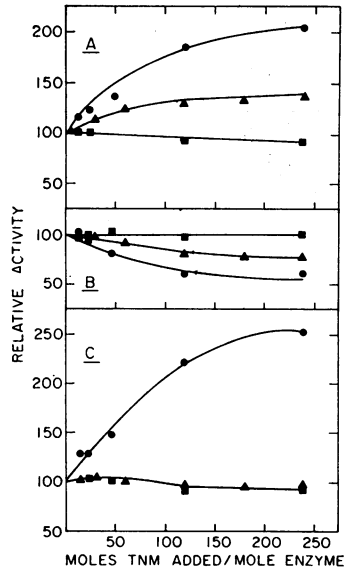

FIG. 2.—The effect of nitration on the catalytic parameters of adenylylated and unadenylylated glutamine synthetase preparation. (A), (B), and (C) refer to the γ-glutamyl transfer, Mg^{2+}-biosynthetic, and Mn^{2+}-biosynthetic activities respectively. The nitration reactions were made as specified in *Materials and Methods*. Reaction mixtures contained 10 mg of $E_{\overline{0.95}}$ (●—●), $E_{7.9}$ (▲—▲), and $E_{\overline{12}}$ (■—■) protein/ml and tetranitromethane (TNM) as indicated. The molecular weight of glutamine synthetase used in the calculations was 600,000.[7] Studies with the $E_{0.8}$ preparation gave results (not shown) similar to those obtained with $E_{\overline{0.95}}$.

shows that as the molar ratio of tetranitromethane to enzyme is increased from 0 to 150, the γ-glutamyl transferase activity (standard assay) increases nearly two-fold, the Mg^{2+}-dependent biosynthetic activity decreases about 40%, and the Mn^{2+}-dependent biosynthetic activity increases about 2.5-fold. Moreover, the effects are nearly maximal when the molar excess of tetranitromethane is 150–200. From this fact and the data in Figure 1, it is assumed that the observed changes are caused by the nitration of 1 to 2 tyrosyl groups per subunit of unadenylylated enzyme. The effects of nitration are qualitatively similar to those produced by adenylylation of glutamine synthetase.[4] Tetranitromethane has no discernible effect on the activity of fully adenylylated enzyme ($E_{\overline{12}}$), and it produced relatively small effects on $E_{\overline{7.9}}$ (Fig. 2).

Effect of nitration on susceptibility to feedback inhibition: The feedback inhibitability of the Mn^{2+}-dependent γ-glutamyl transferase activity of glutamine synthetase is markedly influenced by the extent of adenylylation.[4] This is evident from the data in Table 1 which show that $E_{\overline{0.95}}$ is strongly inhibited by alanine and glycine but is actually stimulated by histidine, tryptophan, AMP, and CTP. On the contrary, $E_{\overline{12}}$ is less sensitive to inhibition by alanine and glycine but is inhibited by all the other effectors. Other data in Table 1 show that nitrated enzyme, prepared by prior incubation of $E_{\overline{0.95}}$ with tetranitromethane, increased the inhibitability of the enzyme by all effectors, whereas nitration had no significant effect on adenylylated enzyme ($E_{\overline{12}}$). Since a 143-fold molar excess of tetranitromethane was used in these experiments, it is assumed that the observed effects are produced by the nitration of 13 tyrosyl groups per mole of enzyme (Fig. 1).

Effects of adenylylation and nitration on the adenine nucleotide specificity of the λ-glutamyl transferase activity and on the enzymes susceptibility to inactivation by urea or Mg^{2+}: The data in Figure 3*A* show that the Mn^{2+}-dependent γ-glutamyl transferase activity of fully adenylylated glutamine synthetase ($E_{\overline{12}}$) is completely inhibited by 60 mM Mg^{2+}, or by 5 *M* urea, whereas neither of these affect the activity of unadenylylated enzyme. Moreover, AMP is unable to replace ADP as a catalytic cofactor for $E_{\overline{12}}$ but it is about 60% as good as ADP in the $E_{\overline{0.8}}$ catalyzed reaction.

The data in Figure 3*B* show that nitration of the enzyme produces responses to

TABLE 1. *Influence of nitration or acetylation on effector responses of glutamine synthetase.*

	Relative Activity					
		Nitrated		Nitrated		Acetylated
Effector	$E_{\overline{0.95}}$	$E_{\overline{0.95}}$	$E_{\overline{12}}$	$E_{\overline{12}}$	$E_{\overline{0.8}}$	$E_{\overline{0.8}}$
Alanine	10	8	23	22	...	...
Glycine	43	35	63	60	...	...
Histidine	113	69	70	66	114	57
Tryptophan	108	77	58	55	113	41
AMP	124	80	25	26	120	68
CTP	148	59	17	18	111	61

γ-Glutamyl transfer assays were as described in *Materials and Methods*, in the presence and absence of 20 mM effector, except that the concentrations of glutamine and hydroxylamine were reduced to 20 mM.[23] Relative activity refers to (activity with effector/activity without effector) ×100. Nitration was performed as described in *Materials and Methods*, using a 143-fold molar excess of tetranitromethane and 20 mg of $E_{\overline{0.95}}$ or $E_{\overline{12}}$/ml. Acetylated $E_{\overline{0.8}}$ was prepared by incubating 9 mg of enzyme with 1.0 ml of 15 mM acetylimidazole as described in *Materials and Methods*.

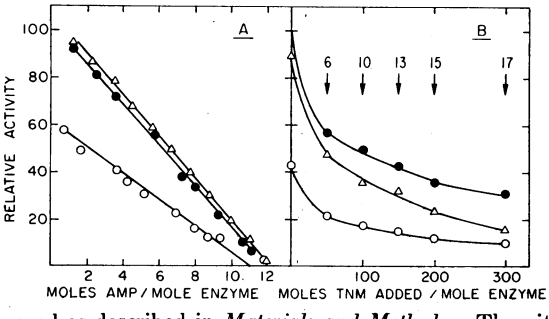

FIG. 3.—The effects of adenylylation or nitration on inhibitability of the γ-glutamyl transferase activity by Mg^{2+} and urea, and on the capacity of AMP to replace ADP in the transfer assay. Enzyme preparations used in part *A* were prepared by the progressive deadenylylation of an $E_{\overline{12.8}}$ preparation by treatment with snake venom phosphodiesterase, as previously described.[3] The enzyme preparations used in part *B* were prepared as described in *Materials and Methods*. The nitration reaction mixtures contained 20 mg protein/ml, and tetranitromethane (TNM) as indicated. The assay mixtures were varied to contain 60 m*M* Mg^{2+} (●—●), 5 *M* urea (△—△), or 4 m*M* AMP in place of ADP (○—○), as indicated. The mixed imidazole transfer assay system was used to determine the effects of Mg^{2+}, in both *A* and *B* and of urea in *A*. In *B* the standard assay system is used to study the effect of urea and the capacity of AMP to replace ADP, whereas the capacity of AMP to replace ADP in *A* was determined in the saturation assay system. In case of the urea and Mg^{2+} experiments, results are expressed as relative activity with the activity of each preparation in the absence of urea or Mg^{2+} set equal to 100. For the experiments in which AMP was substituted for ADP, the activity observed with ADP was set equal to 100. In *B* the numbers over the arrows indicate the equivalents of nitrotyrosyl residues per mole of enzyme, as calculated from the data in Fig. 1.

these three variables that are qualitatively similar to those caused by adenylylation. Nitration of about six tyrosyl groups produces effects that are roughly equivalent to those caused by attachment of six adenylyl groups; however, further nitration is without appreciable effect, whereas further adenylylation leads to additional loss of catalytic activity under these three assay conditions and to complete loss in activity when all 12 subunits are adenylylated.[7,16] Although slightly different assay conditions were used in deriving data from Figures 3*A* and *B*, other studies[20] showed that the observed effects are not related to these differences.

Effects of *O*-tyrosyl group acetylation: Treatment of the unadenylylated enzyme with *N*-acetylimidazole (see *Materials and Methods*) results in a decrease in light absorbancy at 278 mμ, indicating that *O*-acetylation of tyrosyl groups occurs.[9] The data in Figure 4 show that prior incubation of the enzyme with increasing concentrations of *N*-acetylimidazole results in a progressive decrease in

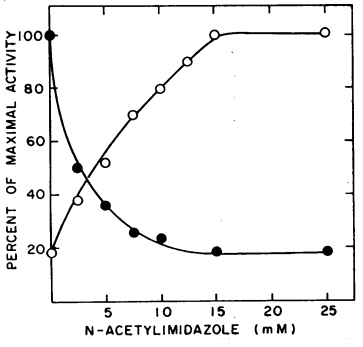

FIG. 4.—Change in divalent cation specificity of glutamine synthetase in response to treatment with increasing *N*-acetylimidazole concentration. Acetylation reactions were performed with the acetylimidazole concentrations indicated at a protein concentration of 9 mg/ml. Activity was measured with the biosynthetic assay using either Mg^{2+} (●—●) or Mn^{2+} ○—○) as the activating cation. To obtain a common ordinate, the results are expressed as percentage of maximal activity observed.

the Mg^{2+}-dependent biosynthetic activity and an increase in the Mn^{2+}-dependent activity. Maximal alteration in both parameters is achieved at 15 mM acetylimidazole. Acetylation of $E_{\overline{0.8}}$ therefore leads to a change in divalent cation specificity that is similar to that provoked by either nitration or by adenylylation of the enzyme.

The similarity in the effects of acetylation, nitration, and adenylylation on the catalytic behavior is further established by the data in Table 2, showing that the γ-glutamyl transferase activity of acetylated $E_{\overline{0.8}}$ is inhibited by Mg^{2+} and is relatively dependent upon ADP; the reaction with AMP is less than 10% that observed with ADP (Fig. 3). The data in Table 2 show also that acetylation of adenylylated enzyme ($E_{\overline{12}}$) has no significant effect on its divalent ion specificity or activity in the presence of Mg^{2+}. It does, however, cause an appreciable decrease in specific activity; this may reflect increased instability of the enzyme after acetylation.

Acetylation mimics nitration and adenylylation also in its effect on feedback inhibition. As shown in Table 1, acetylation of $E_{\overline{0.8}}$ increases its inhibitability by tryptophan, histidine, AMP, and CTP to about the same extent as is achieved by nitration.

Effect of nitration and acetylation on the pH optimum: The γ-glutamyl transferase activity of unadenylylated enzyme has a pH optimum at 7.9, whereas fully adenylylated enzyme has an optimum at pH 6.8.[7] It is therefore of interest that nitration or acetylation of unadenylylated enzyme ($E_{\overline{0.8}}$) causes a decrease in activity at pH 7.9 and an increase in activity at pH 6.8; these changes are thus comparable to those obtained with adenylylation.

Adenylylation and deadenylylation of nitrated and acetylated enzymes: To determine if either nitration or acetylation alters the extent to which glutamine synthetase can be adenylylated, nitrated $E_{\overline{0.8}}$ (containing 15 nitrotyrosyl groups/mole) and acetylated $E_{\overline{0.8}}$ (prepared as described in Table 1) and unmodified $E_{\overline{0.8}}$ were incubated with ^{14}C-ATP and adenylyltransferase as described in *Materials and Methods*. After 2 hr at 37°C, when adenylylation was complete, the reaction was terminated and the amount of covalently bound ^{14}C-AMP was determined. The unmodified enzyme contained 12 equivalents of ^{14}C-AMP/mole. In con-

TABLE 2. *Effect of acetylation on some catalytic properties of glutamine synthetase.*

Enzyme	Specific Activity[a] Mg^{2+}	Specific Activity[a] Mn^{2+}	Relative Activity + 60 mM $MgCl_2$[b]	Relative Activity Replacement of ADP with AMP[c]
$E_{\overline{0.8}}$	120	0.7	100	57
$E_{\overline{0.8}}$ acetylated	22.4	3.7	34.6	6.6
$E_{\overline{12}}$	1.4	12.9	1.9	...
$E_{\overline{12}}$ acetylated	0.6	7.2	1.4	...

[a] The Mn^{2+} and Mg^{2+} biosynthetic assays were used. Specific activity is defined as μmoles of P_i produced/min/mg protein.

[b] γ-Glutamyl transfer activity was measured with the mixed imidazole buffer assay system. Relative activity = (activity with 60 mM $MgCl_2$/activity without $MgCl_2$) × 100.

[c] The standard γ-glutamyl transfer assay was employed to determine the activity first with ADP present and then when ADP was omitted from the reaction mixture and replaced with 4 mM AMP. Relative activity = (activity with AMP/activity with ADP) × 100.

Relative activity in (b) and (c) is expressed as in the legend to Fig. 3. Acetylation of the enzyme preparations was as described for $E_{\overline{0.8}}$ in Table 1.

TABLE 3. *Effect of phosphodiesterase treatment on some catalytic properties of nitrated or acetylated glutamine synthetase.*

Enzyme	Relative Activity				Specific activity
	+ 60 mM $MgCl_2$	+ 5 M Urea	+ 20 mM AMP	+ 20 mM Tryptophan	Mn^{2+}
$E_{\overline{0.8}}$ nitrated	33	13	80	76	2.14
$E_{\overline{12}}$ nitrated	41	16	71	68	1.78
$E_{\overline{0.8}}$ acetylated	50	15	73	67	1.8
$E_{\overline{12}}$ acetylated	52	18	85	81	1.5

γ-Glutamyl transferase activity was measured in the presence and absence of $MgCl_2$ or urea as described in the legend to Fig. 3. The activity in the presence and absence of AMP or tryptophan was determined, and relative activities expressed, as in the legend to Table 1. The Mn^{2+}-biosynthetic specific activity was measured as described in *Materials and Methods* and is defined in the legend to Table 2. Nitration of the glutamine synthetase preparations (20 mg/ml) was performed with a 200-fold molar excess of tetranitromethane. Acetylation of the glutamine synthetase preparations was as described in the legend to Table 1. The chemically modified enzyme preparations were subjected to snake venom phosphodiesterase treatment for 60 min at 37°C as described previously.[3] That the deadenylylation reaction reached completion was determined by measuring the release of ^{14}C-AMP from the chemically modified $E_{\overline{12}}$ ^{14}C-adenylyl glutamine synthetase.

trast, the nitrated and acetylated enzymes contained only 3 and 3.4 equivalents of ^{14}C-AMP/mole respectively. As noted previously the treatment of fully adenylylated glutamine synthetase ($E_{\overline{12}}$) with either tetranitromethane or acetylimidazole does not affect its catalytic properties. However, when nitrated or acetylated $E_{\overline{12}}$ is deadenylylated by treatment with snake venom phosphodiesterase, the resulting enzyme form does not behave like unadenylylated glutamine synthetase; rather, it exhibits properties very similar to those obtained by the nitration or acetylation of an $E_{\overline{0.8}}$ preparation (see Table 3).

Discussion. The alterations in catalytic properties that accompany the nonenzymatic nitration or acetylation of glutamine synthetase are qualitatively analogous to those provoked by the enzyme catalyzed adenylylation of a single tyrosyl group on each subunit of the protein. The spectral changes that accompany the treatment of glutamine synthetase with tetranitromethane or *N*-acetylimidazole indicate that both reagents react also with tyrosyl groups on the enzyme. However, various lines of evidence indicate that these reagents modify tyrosyl residues that are different from the ones involved in adenylylation.

Under comparable conditions, $E_{\overline{0.95}}$ and $E_{\overline{12}}$ are nitrated to the same extent. This contraindicates identity of the adenylylation site with the functionally sensitive nitratable tyrosyl residue since nitration with tetranitromethane involves an ionic mechanism dependent upon ionization of the tyrosyl hydroxyl group.[22,23] Failure of tetranitromethane to nitrate the adenylylated tyrosyl residue is supported by the fact that deadenylylation of nitrated $E_{\overline{12}}$ by treatment with snake venom phosphodiesterase at alkaline pH is not accompanied by an increase in absorbancy at 428 mμ that is characteristic of the unsubstituted ionized nitrotyrosyl group.[24]

The effects of acetylation with *N*-acetylimidazole are less ambiguous since the adenylylated tyrosyl group can certainly not be acetylated; moreover, deadenylylation of adenylylated enzyme does not occur under the conditions of acetylylation. Nevertheless, when the adenylyl groups of acetylated (or nitrated) $E_{\overline{12}}$ are removed by treatment with snake venom phosphodiesterase, the enzyme is converted to a form that is similar to that obtained by acetylation (or nitration) of unadenylylated ($E_{\overline{0.8}}$) enzyme.

From the above considerations, it is evident that the marked changes in catalytic parameters that attend the enzymatic adenylylation of a unique tyrosyl group on each subunit of glutamine synthetase can be elicited also by the chemical modification (nitration or acetylation) of different tyrosyl residues or other as-yet-unidentified functional groups on the enzyme.[25] Although the nonenzymic modifications produce effects that are qualitatively similar to those provoked by adenylylation, their effects are quantitatively less pronounced than those produced by adenylylation.

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[12] $E_{\bar{n}}$ denotes glutamine synthetase preparations with an average of $\bar{n}$ equivalents of covalently bound 5-adenylyl groups per mole of enzyme.

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