MOLECULAR PHARMACOLOGY, 9, 534-541

Physical and Biological Properties of Acetamidino-, β-Dimethylaminopropionamidino-, and Maleyl-L-Asparaginase

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(Received February 12, 1973)

SUMMARY

HARE, LARRY E., AND HANDSCHUMACHER, ROBERT E.: Physical and biological properties of acetamidino-, β-dimethylaminopropionamidino-, and maleyl-L-asparaginase. *Mol. Pharmacol.* **9**, 534-541 (1973).

Modification of *Escherichia coli* L-asparaginase (EC 3.5.1.1) with maleic anhydride, ethyl acetimidate, or methyl β-dimethylaminopropionimidate resulted in enzyme derivatives with altered isoelectric points. Catalytic activity was not significantly changed by these modifications; however, an unexpected covalent cross-linking of enzyme subunits was seen in β-dimethylaminopropionamidino-asparaginase. Although immunological studies *in vitro* indicated minor alterations in cross-reactivity between the native and modified enzymes, no immunological differences were apparent *in vivo*. The biological half-life of these enzymes increases with increasing isoelectric point, and this relationship was associated with enhanced therapeutic effectiveness in leukemic mice.

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) from *Escherichia coli* is useful in producing temporary remissions in some cases of acute leukemia (1, 2). One of the several problems associated with the use of enzymes in therapy is the rapid clearance from plasma after intravenous injection. Of particular importance are the physicochemical properties of the protein as well as its size and state of aggregation (3). Foreign enzymes are also highly antigenic, and repeated therapy often results in rapid clearance accompanied by allergic reactions (4).

Mashburn and Landin (5) attempted to correlate the plasma half-life of L-asparaginase with the isoelectric point of various enzyme preparations. More recently Rutter and Wade (6) reported the rates of clearance of a large number of chemically modified asparaginases from *Erwinia carotovora*. The present study describes the preparation of a series of modified *E. coli* asparaginases with different isoelectric points. Physicochemical, biological, and immunological properties of these enzymes are compared.

This work was supported by grants from the American Cancer Society (IC-64L and PR 27) and the National Cancer Institute (CA10748).

MATERIALS AND METHODS

L-Asparaginase from *E. coli* (Lyovac, Merck Sharp & Dohme, lot C-7941) was provided by the National Cancer Institute. Ethyl acetimidate hydrochloride, butyronitrile, and heptanenitrile were obtained from Eastman Organic Chemicals, and [2,3-^{14}C]-maleic anhydride was a product of Amersham/Searle. Methyl butyrimidate hydrochloride and methyl heptanimidate hydrochloride were prepared as reported by Brooker and White (7). Methyl β-dimethylaminopropionimidate dihydrochloride was

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synthesized from β-dimethylaminopropionitrile (Eastman) by the procedure of Baksheev and Gavrilov (8). Ampholine carrier ampholytes were purchased from LKB Instruments.

Amidination of L-asparaginase (2 mg/ml) was carried out in 0.2 M borate buffer, pH 9.5, at 25° for 6 hr, according to the general method described by Hunter and Ludwig (9). The imidates were present in 10-fold molar excess based on lysine residues in the tetrameric enzyme. Maleylation was performed as described by Butler *et al.* (10). [^{14}C]Maleic anhydride, used to quantitate the maleylation, was diluted with unlabeled maleic anhydride (Aldrich) and sublimed. [^{14}C]Maleic anhydride (0.27 mole/mole of lysyl residues) with a specific activity of 1.2×10^8 cpm/mmole was added to a solution of L-asparaginase (4 mg/ml) in 0.05 M phosphate buffer, and the solution was maintained at pH 8.0 for 30 min in a pH-stat. All enzyme solutions were dialyzed against distilled water to remove excess reagents and were lyophilized and frozen until further use.

Radioactivity in the maleylated enzyme was assayed in a Packard liquid scintillation spectrometer with a toluene-ethanol scintillation fluid containing 1% Cab-O-Sil. Enzyme activity was measured by monitoring the catalytic decomposition of DONV[1] at 274 nm according to the procedure described by Jackson and Handschumacher (11), or by means of the coupled enzymatic assay of Cooney *et al.* (12). Protein concentration was determined by using an extinction coefficient of $E^{1\%}_{278nm} = 7.1$ (13). The number of free amino groups in the modified protein was estimated by the TNBS method of Habeeb (14), except that the assay was performed in 0.1 M borate buffer, pH 9.0, rather than 4% sodium bicarbonate. Unmodified asparaginase was used as a standard. The number of unmodified amino groups which were free to react with TNBS was calculated from the molar extinction coefficient of $E_{333nm} = 1.04 \times 10^4$ determined for the 2,4,6-trinitrobenzene derivative of native asparaginase. The results were expressed as the percentage of the 88 total amino groups unreactive with TNBS (84 lysyl residues and 4 NH_2-terminal leucyl residues). Although this assay was reported to be quantitative with respect to lysyl residues for several proteins, the color yield was nonlinear at asparaginase concentrations above 170 nM. Nevertheless the values obtained from the TNBS reaction were in agreement with those obtained by radioactive labeling with [2,3-^{14}C]maleic anhydride.

Isoelectric focusing was performed in columns of 7% polyacrylamide by modification of the procedure of Righetti and Drysdale (15). The samples were dissolved directly in the gel solution, and polymerization was initiated by the addition of 0.03 mg/100 ml of riboflavin and exposure to light. A constant potential of 100 V was applied to the cooled columns (5 × 65 mm) for 10 hr. To determine the pH gradient, gels containing no sample were sliced in 1-mm sections and allowed to stand in distilled water (0.4 ml) for at least 2 hr. The sample gels were washed extensively with 10% trichloracetic acid to remove ampholine buffers and stained with 0.05% Coomassie blue in 10% trichloracetic acid. After destaining in an EC apparatus, the gels were preserved in 5% aqueous glycerol and the band intensity was determined with a Gilford spectrophotometer scanning attachment at 650 nm. SDS-polyacrylamide electrophoresis was performed as previously described (16).

The degree of dissociation of asparaginase into subunits was determined by ultracentrifugation in a Beckman L2-65 ultracentrifuge on 5–20% sucrose gradients (17). The gradients were subjected to a force of 200,000 × *g* for 14 hr and then divided into 25-drop fractions. Protein was determined by absorption at 278 nm, and the relative positions of tetrameric asparaginase (mol wt 133,000) and monomeric asparaginase (mol wt 33,000) were established with standard protein molecular weight markers. The enzyme activity of each species was measured by means of the DONV assay.

Animal studies were conducted in AKD_2F_1 mice (Jackson Laboratory) bearing L5178Y

[1] The abbreviations used are: DONV, 5-diazo-4-oxo-L-norvaline; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SDS, sodium dodecyl sulfate; DMAP, β-dimethylaminopropionamidino.

asparaginase-sensitive tumors propagated as described by Summers and Handschumacher (18). In the blood clearance studies, asparaginase preparations (200 units/kg) were injected intravenously in 0.15 M NaCl on the sixth day after intraperitoneal implantation of 10^5 L5178Y cells. Orbital blood samples (0.1 ml) were centrifuged at 7500 rpm for 10 min, and the plasma was assayed for asparaginase activity. In the survival experiments mice were treated with intraperitoneal L-asparaginase preparations (200 units/kg) 3, 5, and 7 days after implantation of 10^6 L5178Y cells. Immunological studies were carried out according to the methods described by Peterson, Handschumacher, and Mitchell (19).

RESULTS

The properties of the amidinated and maleylated asparaginases are tabulated in Table 1. Acetamidino-, DMAP-, and maleyl-asparaginase retained at least 70% of the original asparaginase activity; however, the number of lysine residues modified was different in each case. Butyramidino- and heptanamidino-asparaginase retained 88% and 20% of the original activity, respectively, when 60% of the lysyl residues of each were amidinated; further treatment with heptanimidate caused enzyme precipitation. Preliminary biological results indicated that butyramidino- and acetamidino-asparaginase exerted nearly identical effects; therefore only the latter derivative was studied extensively. The V_{max} and K_m values of the modified enzymes were similar to the values obtained for L-asparaginase (Table 1). In addition, the relative activities of the various enzymes with respect to the alternative substrates glutamine and DONV were unchanged. Ultracentrifugation on sucrose gradients indicated that greater than 40% maleylation of asparaginase resulted in dissociation into subunits which did not retain enzymatic activity. However, the maleylated species used in the biological studies contained only 15% modified lysyl residues and was tetrameric. Extensive amidination of asparaginase by the imidates described above did not cause subunit dissociation.

Isoelectric focusing on polyacrylamide gels (Table 1 and Fig. 1) indicated that the enzyme derivatives possessed modified isoelectric points, as expected. The pI of the acetamidino derivative was similar to that of native asparaginase, while that of maleyl-asparaginase was 1 pH unit lower. DMAP-asparaginase separated into two peaks, corresponding to isoelectric points of 6.1 and 6.9, the latter being the major species. The shoulders evident in the maleyl-asparaginase peak suggest that this derivative is also slightly heterogeneous. The asymmetry in the peaks of native and acetamidino-asparaginase was due to diagonal skewing of these bands in the gels.

Polyacrylamide gel electrophoresis indicated that DMAP-asparaginase was more highly aggregated than the other preparations. These data suggested that an unexpected cross-linkage of subunits may have occurred during the amidination reaction. Electrophoresis on SDS-polyacrylamide gels verified the existence of covalent cross-linkage between monomers of the enzyme (Fig. 2). The mobility of the major band of DMAP-asparaginase on SDS-polyacryl-

TABLE 1

Properties of native and modified L-asparaginase

Enzyme derivative	Catalytic activity	Catalytic activity as percentage of control	K_m	Lysine modification		pI	Initial clearance rate ($t_{1/2}$)
				TNBS	^{14}C		
	units/mg	%	*M* × 10^5	%	%		*hr*
L-Asparaginase	250		1.6			4.9	2.0
Acetamidino-L-asparaginase	204	81	1.5	77		4.6	1.8
DMAP-L-asparaginase	213	85	1.7	41		6.1, 6.9	2.3
Maleyl-L-asparaginase	170	69	2.3	13	15	4.0	1.3

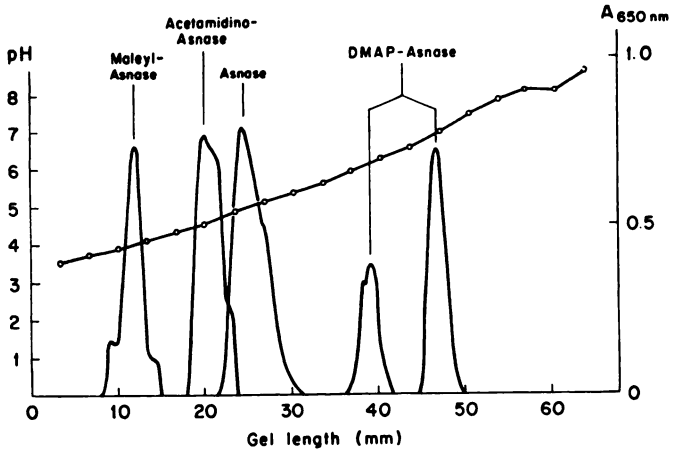

FIG. 1. *Isoelectric focusing of native and modified asparaginase on polyacrylamide gels*
This figure represents a composite of gel scans from two experiments. The pH gradient represents an average of two gels which contained nearly identical gradients.

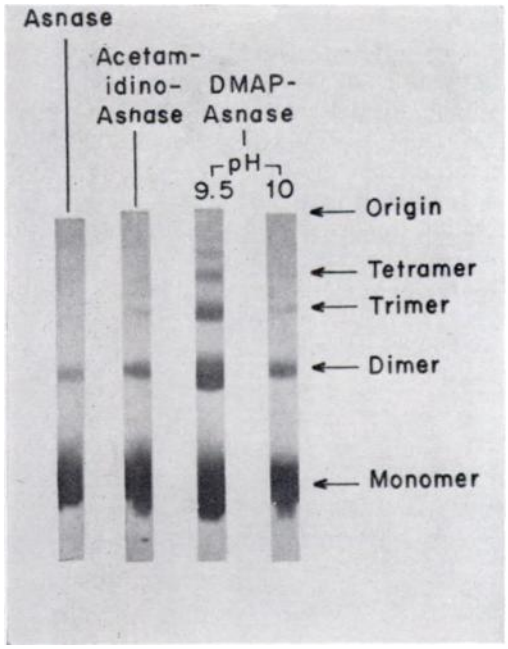

FIG. 2. *SDS-polyacrylamide gel electrophoresis of native and modified asparaginase*

amide gels was identical with that of the monomer of L-asparaginase, while the remaining three bands possessed mobilities corresponding to dimer, trimer, and tetramer. When the amidination was performed at pH 10, rather than 9.5, less cross-linkage occurred (Fig. 2), and the species with an isoelectric point of 6.1 nearly disappeared. The derivative obtained at pH 10 possessed the same amount of enzyme activity as the pH 9.5 derivative. Since both species of DMAP-asparaginase possessed isoelectric points considerably greater than that of L-asparaginase, subsequent biological studies were performed with the mixture.

The clearance rates for the various modified asparaginases from leukemic mice are depicted in Fig. 3. All species exhibited an initial rapid clearance rate during the first 3 hr; however, activity persisted in the blood for more than 24 hr. The biological half-lives (Table 1) were calculated by the method of least squares from the initial portions of the time curves. The rate of disappearance of enzyme activity from the blood decreased with increasing pI. In an experiment with nonleukemic mice, more rapid clearance of unmodified asparaginase occurred over 24 hr.

The same relationship was seen in survival studies of mice implanted with L5178Y cells (Fig. 4). The more rapidly cleared maleyl-asparaginase was less effective in increasing survival times than were the other derivatives, while the most positively charged and most slowly cleared species, DMAP-asparaginase, increased the mean survival time 2 days longer than L-asparaginase in these studies.

The altered isoelectric points of these enzymes were also associated with altered immunoreactivity. Inhibition of passive hemagglutination titers of the modified enzymes against specific antibodies for L-asparaginase (Fig. 5) indicated that maleyl-asparaginase competed as well as L-asparaginase for the antibodies; however, the amidinated derivatives were less effective than L-asparaginase.

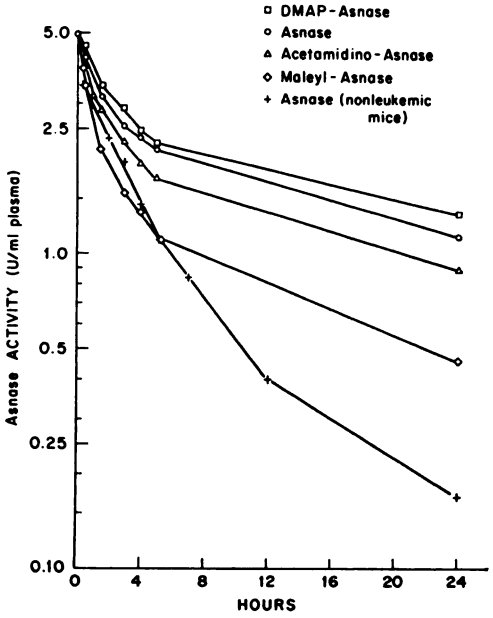

FIG. 3. *Rates of clearance of native and modified asparaginase from mice implanted with L5178Y cells*

Each animal received 5 units of enzyme activity in 0.1 ml of 0.15 M NaCl. The data represent the average of groups of three mice. A control group of animals not bearing the tumor was also included.

Extension of these results to a system *in vivo* was attempted by preparing asparaginase-immune mice as described by Vadlamudi *et al.* (20). Large intravenous doses of L-asparaginase activity (2000 units/kg) were cleared in less than 1 min by these mice. The modified enzymes were also removed from the plasma within 1 min. Thus differences in cross-reactivity among the enzyme derivatives *in vitro* were not apparent *in vivo*.

DISCUSSION

Extensive succinylation of L-asparaginase has been demonstrated by Shifrin and Grochowski (21) to cause dissociation of L-asparaginase from *E. coli* into subunits, with concurrent loss of enzymatic activity. We have observed that maleylation also causes subunit dissociation. However, low degrees of modification by maleic anhydride resulted in retention of most of the enzymatic activity without affecting quaternary structure. This modification caused a considerable reduction in the isoelectric point of the enzyme.

Amidination was selected as an alternative modification of lysine residues, since this type of reaction minimally affects protein conformation or catalytic activity (22). Fur-

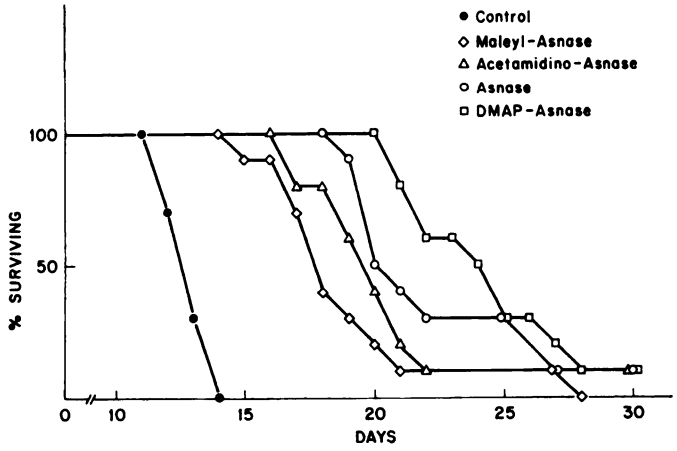

FIG. 4. *Effect of native and modified L-asparaginase on survival of mice implanted with L5178Y cells*

The treatment schedule is described under MATERIALS AND METHODS. Control animals received 0.15 M NaCl. The differences in time of death of groups of 10 mice treated with asparaginase or modified asparaginase were significant at $p < 0.1$.

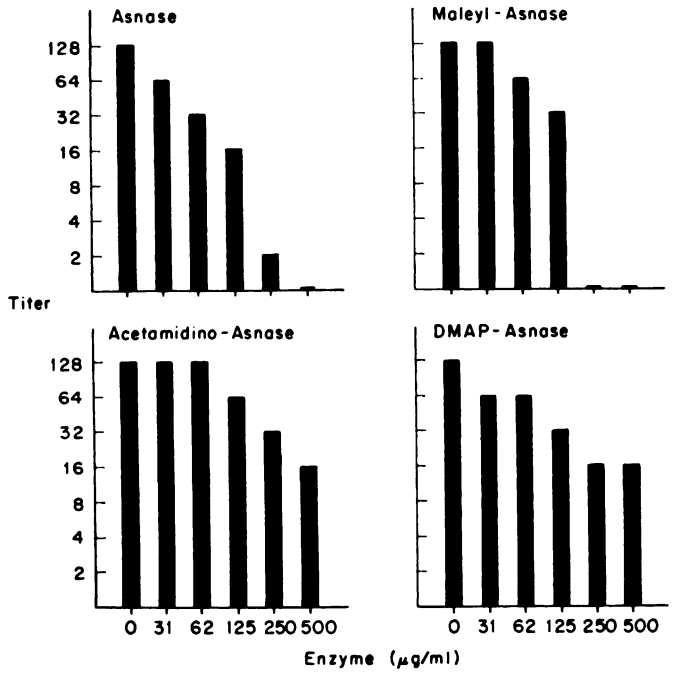

FIG. 5. *Inhibition of passive hemagglutination titer of anti-E. coli asparaginase rabbit serum previously incubated with native or modified asparaginase*

Diluted anti-asparaginase rabbit serum was first incubated with solutions of native and modified L-asparaginase and serially diluted with normal rabbit serum in microtrays. Formalinized goat red blood cells previously sensitized to *E. coli* L-asparaginase were added to all wells, and the trays were incubated in the cold overnight. The titers were compared with those obtained when antiserum was not first incubated with asparaginase (19).

thermore, it is possible to incorporate different functional groups into the imidate and thus obtain a variety of derivatives. The reagent β-dimethylaminopropionimidate, first described by Hunter and Ludwig (9), caused a large change in the isoelectric point of asparaginase without significantly destroying enzymatic activity. Extensive modification of the lysyl residues with acetimidate did not have adverse effects upon asparaginase activity, but the introduction of alkyl chains longer than *n*-butyl resulted in inactive enzyme. None of the amidination reactions caused dissociation of the tetrameric enzyme into subunits.

Cross-linkage of asparaginase subunits has previously been accomplished by the use of dimethylsuberimidate (16), but was unexpected for the monoimidate, β-dimethylaminopropionimidate. Such cross-linkage can be explained by the mechanism proposed by Hand and Jencks (23) for the reaction of imido esters with amines. These authors proposed a tetrahedral addition intermediate which could decompose to a monosubstituted amidine. Alternatively, at more acidic pH values, ammonia might be lost and a new imidate incorporating the protein lysyl residues would be formed. This imidate could then react with an additional lysine amino group to form a cross-linked species. At the relatively high pH used in the reaction with asparaginase, cross-linkage should be a minor reaction; however, the pH-rate profile for each reaction is influenced by the functional groups in the alkyl portion of the imidate. The occurrence of a small amount of intrasubunit cross-linkage with acetimidate might explain the slightly lower isoelectric point for this species in comparison with native asparaginase. Cross-linkage of DMAP-asparaginase would also reduce the total change in net charge obtained in the modification. Thus the major species (pI 6.9) may represent non-cross-linked material while the minor species (pI 6.1) is probably cross-linked

subunits. This conclusion is strengthened by the following observations. Approximately 30% of the total protein existed in cross-linked form (Fig. 2), and the lower isoelectric species also represented 30% of the protein. Furthermore, the species with the lower isoelectric point nearly disappeared when the reaction was carried out at pH 10 rather than 9.5—conditions which resulted in less cross-linkage as observed in SDS gels. Since the retention of enzymatic activity was identical in reactions carried out at pH 9.5 or 10, both isoelectric species of DMAP-asparaginase would appear to be active.

These chemical modifications of asparaginase affected the rate of clearance from plasma after intravenous injection. Initial biological half-lives of about 2 hr were observed, and subsequent clearance occurred with approximately a 24-hr half-life. Riley (24) reported a $t_{1/2}$ of 25 hr for L-asparaginase in mice infected with a lactate dehydrogenase-elevating virus, and a value of 4 hr for uninfected animals. It is assumed that the L5178Y tumor line used in these experiments is infected with the lactate dehydrogenase-elevating virus; thus the initial rapid clearance rate may reflect the time required for tissue distribution. Confirmation of this view can be seen in the clearance of unmodified asparaginase in nonleukemic mice. The initial rate was essentially the same as that in leukemic animals. However, residual activity after 24 hr was only 0.17 unit/ml of plasma, compared to 1.13 units/ml of plasma in leukemic animals.

Several factors have been postulated as determinants in the clearance of proteins from plasma. Many studies have demonstrated that aggregated proteins are rapidly removed from the circulation, presumably by phagocytosis. Other studies, using modified serum proteins, have suggested that rate of clearance is proportional to net charge on the protein at physiological pH (3). However, studies by Mashburn and Landin (5) with different unmodified preparations of L-asparaginase from *E. coli* indicated that pI values below 5 were associated with the slowest clearance. Rutter and Wade (6) have reported on a series of chemically modified L-asparaginase preparations from *Erwinia carotovora*. In their studies with this enzyme, which has a pI of 8.7 in the native state, plasma clearance was slowest with preparations having an isoelectric point between 5 and 6. Our results indicate that the longest half-life was associated with DMAP-asparaginase (pI $\cong$ 7), which would possess a minimal charge at physiological pH. This is surprising, since a significant proportion (30%) of this modified enzyme exists in aggregated multiples of the tetrameric structure. These diverse results reflect what must be a very complex series of factors responsible for plasma clearance of a foreign protein. Until much more is known about the chemical and physical factors which affect this process, further speculation seems inappropriate.

Prolongation of life in the leukemic animals correlated with half-life in plasma. The more rapidly cleared maleyl-asparaginase was less effective than native enzyme, while the prolonged half-life of DMAP-asparaginase resulted in an increase in mean survival time over native enzyme. It remains to be determined whether or not there is a cause-effect relationship between these parameters. Thus simple chemical modification of L-asparaginase does show promise as a means of improving therapeutic efficacy. Wagner *et al.* (25) previously demonstrated that partially deaminated L-asparaginase is as active as the native enzyme. Studies in humans indicated that the elimination rate from serum of this derivative is markedly diminished (26).

Although the antigenic differences observed *in vitro* by inhibition of hemagglutination were too small to be significant *in vivo*, they did indicate that other alterations of lysine residues might enable one to change the antigenicity of asparaginase without altering enzymatic activity. Such results have been described for trypsin, which retains catalytic activity but loses immunological reactivity after polyalanylation of amino groups (27).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Mrs. Celeste Grant and the collaboration of Mr. Robert Peterson on portions of this work.

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