

# Analytical Letters

Part A—Chemical Analysis

Part B—Clinical and  
Biochemical Analysis

REDUCTIVE ISOPROPYLATION OF AMINO GROUPS IN LYSINE CONTAINING  
PEPTIDES

Key Words: peptides; isopropyllysine; reductive alkylation;  
 $\alpha$ -N-isopropylamino; electrophoresis; dansylation

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ABSTRACT

Model peptides, Gly-Gly-Lys-Arg, Arg-Lys-Asp-Val-Tyr, and Pro-Gly-Lys-Ala-Arg were reductively alkylated with [<sup>2</sup>H<sub>6</sub>]acetone and sodium borohydride to assess the effects on peptide behavior. Lysine residues were converted to  $\epsilon$ -N-isopropyllysine which eluted between phenylalanine and histidine on amino acid analysis. Amino terminal groups were also modified to an extent which depended on the particular peptide (glycine 100%, arginine 30%, and proline 10%-20%). High voltage paper electrophoresis of native and isopropylated peptides showed similar properties except for minor decreases in the mobility of the modified

peptides due mainly to increased molecular weight. Isopropyl-lysine was not an effective substrate for trypsin, and  $\alpha$ -N-isopropyl-amino acids did not form dansyl chloride derivatives. These findings should aid in the location, by peptide mapping techniques, of specific modified residues in reductively isopropylated proteins.

### INTRODUCTION

Reductive alkylation of amino groups is a useful way to introduce isotopic labels, such as  $^2\text{H}$ ,  $^{13}\text{C}$ , or  $^{14}\text{C}$ , into proteins and peptides. By judicious choice of alkylating and reducing agent, one can specify the location on the amino group of the covalently bound probe. Means and Feeney<sup>2</sup> developed a general alkylation technique using aliphatic aldehydes or ketones with sodium borohydride as the reducing agent. Most recent work has focused on reductive methylation in an effort to make the probe as nonperturbing as possible; unfortunately the result is a mixture of  $\epsilon$ -N-monomethyl- and  $\epsilon$ -N,N-dimethyllysine. Reductive methylation causes little change in the physiochemical properties of a protein<sup>2</sup>, although it does reduce the susceptibility of the protein to tryptic hydrolysis<sup>3,4</sup>. Additionally, if a modified lysine residue is part of the active site, as in ribonuclease<sup>2</sup>, it may affect enzymatic activity.

Deuterium is a useful label for NMR, IR, and neutron diffraction studies. A high level of deuterium labelling can be

achieved by using [ $^2\text{H}_6$ ]acetone and sodium borohydride to reductively alkylate amino groups in peptides and proteins according to the reaction scheme in Fig. 1. In contrast to reductive methylation, only the monoisopropyl derivative is formed<sup>5</sup>. The modified peptide or protein is thus labelled with six equivalent, nonexchangeable deuterium atoms on each alkylated amino group. In addition to N- $\epsilon$ -isopropyllysine residues, N- $\alpha$ -isopropyl derivatives of the amino terminal residues were formed to an extent, depending on the nature of the peptide and its amino terminal residue<sup>6</sup>. The  $\text{pK}_a$  of a monoalkylated lysine residue is about 0.1 pH unit higher than that of a lysine residue<sup>7</sup>, so the overall charge on a peptide is not greatly changed, and the spatial distribution of charges is maintained.

While the particular peptides used in this study were chosen primarily because they contain nonterminal lysine residues, two of them illustrate the types of peptide sequences which are of pharmacological interest. For example, Arg-Lys-Gly-Val-Tyr, constitutes the active site of thymopoietin, and is synthesized for possible therapeutic use<sup>8</sup>. Pro-Gly-Lys-Ala-Arg contains the amino terminal proline, which though not common in proteins, does occur in some toxins<sup>9</sup>. We have examined the effects of peptide structure on the availability of an amino group for alkylation, the effects of alkylation on the physicochemical behavior of the peptide, and its sensitivity to hydrolysis by trypsin.

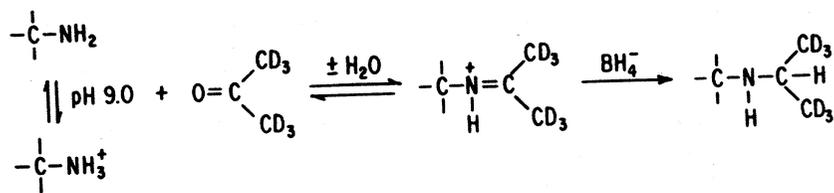


FIG. 1. Deuterium labelling by reductive isopropylation.

#### MATERIALS AND METHODS

Glycyl-glycyl-L-lysyl-L-arginyl diacetate (Gly-Gly-Lys-Arg) and dimethyllysine were purchased from Vega Biochemicals<sup>10</sup> (Tucson, AZ), L-arginyl-L-lysyl-L-aspartyl-L-valyl-L-tyrosine acetate (Arg-Lys-Asp-Val-Tyr) and free amino acids from Calbiochem-Behring (La Jolla, CA), L-prolyl-glycyl-L-lysyl-L-alanyl-L-arginine 1/2 H<sub>2</sub>SO<sub>4</sub> (Pro-Gly-Lys-Ala-Arg) from Serva (Accurate Chemicals, Hicksville, NY), and poly-L-lysine HBr from Sigma (St. Louis, MO). The actual compositions of the peptides were determined by amino acid analysis. All of the peptides were essentially free of contaminating amino acids. Nevertheless, all samples were chromatographed through Sephadex G-10 with 0.01 M acetic acid to remove any contaminating salts.

#### Preparation of [<sup>2</sup>H<sub>6</sub>]Isopropyl Labelled Peptides and Amino Acids

Reductively alkylated peptides were prepared by the method of Fretheim et al.<sup>5</sup>, modified as follows for use with small peptides. To a solution of 5 mg peptide in 0.5 mL 0.2 M borate (pH 9.0, 20% p-dioxane) was added 0.25 mL [<sup>2</sup>H<sub>6</sub>]acetone (99.7 atom % <sup>2</sup>H, KOR Isotopes, Cambridge, MA), making the final solu-

tion 6.4 M in acetone. Small quantities of  $\text{NaBH}_4$  (98%, Alfa Products, Danvers, MA) to a total of 14 mg (530 mM) were added with stirring over a 15-minute period. The mixture was stirred for an additional 15 minutes, then titrated to pH 4.5 with 0.5 M HCl. Excess reagents were removed by gel filtration through a 0.9 X 50 cm column of Sephadex G-10 with 0.01 M acetic acid. The column effluent was monitored at 206 nm, and fractions containing peptide were lyophilized. In the case of free amino acids, no attempt was made to remove excess reagents.

#### Amino Acid Analyses

Unmodified and alkylated peptides were hydrolyzed at 105°C for 24 hours with 5.7 N HCl containing phenol (0.05%) in sealed evacuated tubes. Analyses were performed on a Beckman 119 CL Amino Acid Analyzer using the standard 90-minute single column hydrolyzate protocol. Specifically, this procedure employs a 0.6 X 22 cm column of W3-H resin eluted with the following buffers: pH 3.25, 0.20 N  $\text{Na}^+$ ; pH 3.95, 0.40 N  $\text{Na}^+$ ; pH 6.4, 1.00 N  $\text{Na}^+$ . Other details include a 50-65°C temperature change at 20 minutes, and the use of ninhydrin formulated with dimethyl sulfoxide for detection. Results were obtained as nmol per sample, and are reported as molar ratios. Intact peptides were also analyzed without hydrolysis to check for contaminating amino acids or breakdown products.

#### Proteolysis by Trypsin

A freshly prepared solution of trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK-trypsin from

Worthington Biochemicals, Freehold, NJ) at pH 8.2 was added to a solution of 3 mg peptide in 1.5 mL water (pH 8.2) so that the trypsin:peptide ratio was 1:50. Proteolysis was carried out in small, stoppered culture tubes under N<sub>2</sub> at 37°C. After 1.5 hours the pH, which remained constant, was checked, a second equal aliquot of trypsin was added, and incubation continued for an additional 1.5 hours. The peptide fragments were recovered by lyophilization.

#### Identification of Amino Terminal Residues

Amino terminal residues of peptides and peptide fragments were identified by the method of Weiner et al.<sup>11</sup>. The peptides and their tryptic digests were dansylated, then hydrolyzed in 5.7 N HCl at 105°C for 16 hours. The hydrolyzates were dried under vacuum, extracted with ethyl acetate, and chromatographed in two dimensions on polyamide plates. Solvent I (1.5% formic acid in water) and Solvent II (benzene:acetic acid, 9:1) were used according to the method of Woods and Wang<sup>12</sup>. The chromatograms were compared under uv light with a standard amino acid mixture.

#### Electrophoresis

High-voltage paper electrophoresis of native and reductively alkylated peptides and their tryptic digests was carried out at 40 V/cm for 1.5 hours in pH 6.4 buffer as described previously<sup>13</sup>. The electrophoretograms were cut in strips and stained with ninhydrin to identify amino groups, with phenanthrenequinone to locate arginine residues, and with  $\alpha$ -nitroso- $\beta$ -naphthol to locate

tyrosine residues. For each experiment, six samples were applied to the paper: the native peptide, reductively isopropylated peptide, tryptic digests of native and alkylated peptides, lysine, and arginine.

## RESULTS AND DISCUSSION

### Amino Acid Analyses

Table 1 lists the relative proportions of unmodified amino acids in the native and reductively isopropylated peptides. With the exception of proline, the amino acid analyses of the native peptides agreed with their expected stoichiometry. One possible explanation for the low proline value is the incomplete removal of a blocking agent used in the commercial synthesis of Pro-Gly-Lys-Ala-Arg.

Because  $\alpha$ -N-isopropylglycine and  $\alpha$ -N-isopropylarginine, prepared from the free amino acids, were not detected in the chromatogram from the amino acid analysis, and the ninhydrin color yield for  $\epsilon$ -N-isopropyllysine, prepared from poly-L-lysine, was significantly less than that for lysine, these amino acids were quantitated by difference. In our system,  $\epsilon$ -N-isopropyllysine eluted with the third buffer, well separated in the area after phenylalanine and before the first basic amino acid, histidine.  $\alpha$ -N, $\epsilon$ -N-Diisopropyllysine eluted between histidine and lysine, as did the comparable dimethyllysine. Identical elution positions were obtained for isopropyllysine and [ $^2\text{H}_6$ ]isopropyllysine. No modifications in the standard protein

TABLE 1

Molar Ratios of Unmodified Amino Acids in Peptides  
and Reductively Isopropylated Peptides

Amino acid	Peptide	Modified peptide
Gly-Gly-Lys-Arg		
Gly	1.85 <sup>a</sup> (2) <sup>b</sup>	0.96 (1)
Lys	1.00 (1)	0.05
Arg	1.00 (1)	1.00 (1)
Pro-Gly-Lys-Ala-Arg		
Pro	0.62	0.44
Gly	1.06 (1)	1.12 (1)
Lys	0.90 (1)	0.10
Ala	1.06 (1)	0.96 (1)
Arg	0.99 (1)	0.92 (1)
Arg-Lys-Asp-Val-Tyr		
Arg	1.00 (1)	0.70
Lys	1.00 (1)	0.21
Asp	1.06 (1)	1.04 (1)
Val	0.95 (1)	0.96 (1)
Tyr	1.00 (1)	1.01 (1)

<sup>a</sup> Average of at least three separate determinations.

<sup>b</sup> Rounded to show agreement with stoichiometry.

hydrolyzate analysis procedure were required to obtain the separations in contrast to the dedicated methodology catalogued in a recent review of lysine derivatives<sup>14</sup>.

With small peptides, one would predict complete alkylation of both  $\alpha$ -amino and  $\epsilon$ -amino groups. In the case of Gly-Gly-Lys-Arg, this prediction was substantiated. With Pro-Gly-Lys-Ala-Arg, the lysine residue was completely isopropylated; in addition, a small peak due to  $\alpha$ -N, $\epsilon$ -N-diisopropyllysine was detected, indicating that this peptide was partially cleaved during the reaction. Proline was modified to a lesser extent than other amino terminal acids as might be expected, because of the presence of an imino rather than a primary amino nitrogen. Reductive reactions of the imino nitrogen involve cleavage of the proline ring and in this case, resulted in a product which eluted in the amino acid analysis near the position of serine. The behavior of Arg-Lys-Asp-Val-Tyr was most interesting, in that neither lysine nor the amino terminal arginine was fully modified. To examine the possibility of steric constraints, we constructed a space-filling model of this peptide. It was apparent that readily accessible conformations existed in which either the  $\epsilon$ -amino portion of lysine or the  $\alpha$ -amino portion of arginine was in close proximity with the carboxyl group of aspartic acid leading to possible ionic interactions between these residues. When an isopropyl group was added to either residue, spatial constraints favored the conformation in which the unmodified residue interacted with the carboxyl group of aspartate. Marsh et al.<sup>15</sup> found that the

amino terminal groups in insulin were reductively methylated more readily than lysine; the opposite finding here may reflect a preference of the native peptide for the conformation in which arginine and aspartate are ion paired as recently reported by Tourwe et al.<sup>16</sup>. In the three peptides examined lysine and amino terminal residues were affected by reductive isopropylation while proportions of other amino acids remained constant.

#### Amino Terminal Residue

Amino terminal residues of the peptides, reductively isopropylated peptides, and tryptic digests were determined as their dansyl derivatives. The results are summarized in Table 2.  $\alpha$ -N-Isopropyl amino acids did not form derivatives with dansyl chloride. The absence of dansyl amino acids from tryptic digests of the reductively alkylated peptides is strong evidence that  $\epsilon$ -N-isopropyllysine is not an effective substrate for trypsin. In methylated proteins, cleavage of peptide bonds on the carboxyl side of  $\epsilon$ -N,N-dimethyllysine was not catalyzed by trypsin<sup>3,4</sup>, while those adjacent to  $\epsilon$ -N-monomethyllysine were less resistant<sup>3</sup>. Isopropyllysine is comparable in size to dimethyllysine, and this rather than the number of substituents appears to be the controlling factor.

#### Electrophoresis

Electrophoretic patterns of native and modified peptides and their tryptic digests are shown in Figs. 2-4. Although amino acid analysis, based on the concentration of unmodified amino acids in a peptide was used to assure that samples for

TABLE 2  
Amino Terminal Residues

Peptide	Treatment	Terminal residues
Gly-Gly-Lys-Arg	none	Gly
"	r. i. <sup>a</sup>	-
"	t. d. <sup>b</sup>	Gly, Arg
"	r. i., t. d.	-
Pro-Gly-Lys-Ala-Arg	none	Pro
"	r. i.	-
"	t. d.	Pro, Ala, Arg (vw) <sup>c</sup>
"	r. i., t. d.	-
Arg-Lys-Asp-Val-Tyr	none	Arg
"	r. i.	Arg (weak)
"	t. d.	Arg, Asp
"	r. i., t. d.	-

<sup>a</sup> Reductively isopropylated.

<sup>b</sup> Digested with trypsin.

<sup>c</sup> Very weak.

electrophoresis were of comparable size, the ninhydrin tests obtained with unmodified peptides were always more intense than those for isopropylated peptides. Weak unanticipated bands were seen in the electrophoretograms of the unmodified peptides. These were apparently due to electrophoretic breakdown, because

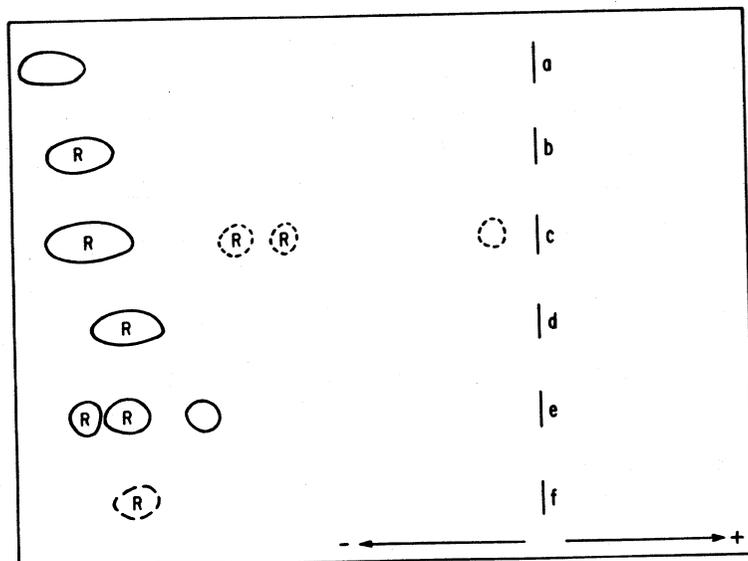


FIG. 2. Electrophoretograms for Gly-Gly-Lys-Arg (G-G-K-R)<sup>18</sup>. The samples are: a) lysine, b) arginine, c) native peptide, d) reductively isopropylated peptide, e) tryptic digest of the native peptide, f) tryptic digest of the isopropylated peptide. Bands outlined with solid lines (—) gave intense ninhydrin positive tests, dashed lines (---) indicate weak ninhydrin tests, and dotted lines (· · ·) indicate very weakly ninhydrin positive spots. Other positive tests for specific amino acids are indicated by labelling the band with the single letter amino acid code<sup>17</sup> for the residue. Conditions for the electrophoresis may be found in the text under Materials and Methods.

there was no evidence of extra amino acids or peptide fragments in the amino acid analyses of the peptides either with or without hydrolysis. In addition, these extra bands could not be predicted by dansylation, a technique which is generally more sensitive than the detection methods used with electrophoresis.

Gly-Gly-Lys-Arg (Fig. 2) migrated primarily as a dicationic species at pH 6.4. Three very minor bands were also seen; one

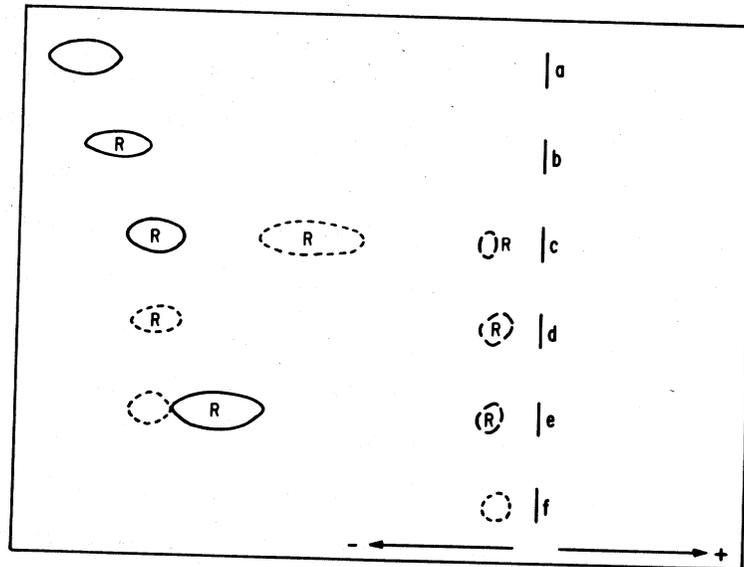


FIG. 3. Electrophoretograms for Pro-Gly-Lys-Ala-Arg (P-G-K-A-R)<sup>17</sup>, for details, see the legend to Fig. 2.

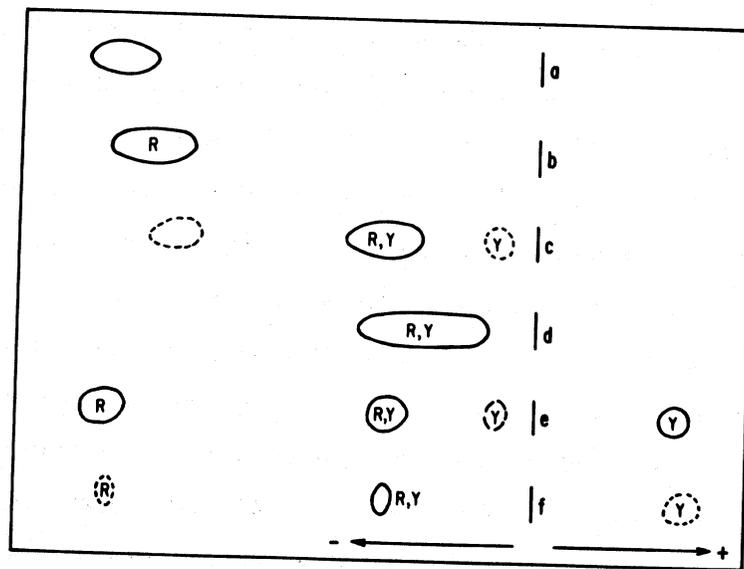


FIG. 4. Electrophoretograms for Arg-Lys-Asp-Val-Tyr (R-K-D-V-Y)<sup>17</sup>, for details, see the legend to Fig. 2.

which migrated as a neutral peptide and did not respond in the arginine test is either free glycine or the dipeptide Gly-Gly. Two other minor bands with 67% and 56% of the mobility of the intact peptide did form fluorescent complexes with phenanthrene-quinone. Reductively isopropylated Gly-Gly-Lys-Arg migrated as a single species at 90% of the rate of the native peptide, this appears to be an effect of the 20% increase in weight when this small peptide is reductively isopropylated. The tryptic digest of the native peptide migrated on electrophoresis as three species corresponding to Gly-Gly-Lys-Arg, Lys-Arg, and Gly-Gly-Lys. In contrast, the tryptic digest of the modified peptide showed only undigested isopropylated peptide.

Pro-Gly-Lys-Ala-Arg (Fig. 3) migrated primarily as a dicationic species, though not as far as Gly-Gly-Lys-Arg because of its greater weight. There was also a weak neutral band, presumably Pro-Gly or proline, and a still weaker monocationic band which formed a fluorescent complex with phenanthranene-quinone. The reductively isopropylated peptide was significantly less soluble than the native peptide, and much of the material remained at the origin, though minor bands which migrated at a rate comparable to those of the native peptide were detected. The tryptic digest of the native peptide migrated as three bands, the native peptide, a neutral band, and a monocationic band. The tryptic digest of the modified peptide was generally insensitive to the various tests, showing only a weakly positive ninhydrin spot in the neutral band.

Arg-Lys-Asp-Val-Tyr (Fig. 4) migrated as a monocationic species giving positive tests for arginine and tyrosine. Two very faint additional spots were seen; one in the neutral band giving a positive tyrosine test, either free tyrosine, Val-Tyr, or Lys-Asp-Val-Tyr, and the other either a dicationic peptide or a single positively charged amino acid. Since the arginine test was negative, it is not certain what the origin of this spot might be. Despite the incomplete alkylation of this peptide, arginine (30%), and lysine (80%), the modified peptide migrated as a single, albeit broadly smeared spot moving with slightly less mobility than the native peptide. The tryptic digest of the native peptide migrated as three major spots corresponding to whole peptide, the dicationic species Arg-Lys, and the anionic species Asp-Val-Tyr. There was also a minor band in the neutral region. Alkylated peptide treated with trypsin migrated primarily as the whole modified peptide with minor bands corresponding to Arg-Lys and Asp-Val-Tyr, suggesting some tryptic digestion due to the incomplete isopropylation.

Reductive isopropylation with [ $^2\text{H}_6$ ]acetone and  $\text{NaBH}_4$  results in the addition of a probe, comprising a single isopropyl group containing six equivalent deuterons, to each accessible primary amine. The extent of this reaction can be easily quantitated and modified residues identified by amino acid analysis. In small peptides, interactions between neighboring residues are stabilized by ionic or hydrogen bonds. This secondary structure may impose spatial constraints that limit access of the isopropyl group.

Therefore, the degree to which  $\alpha$ - and  $\epsilon$ -amino groups can be reductively isopropylated, conveniently determined by this method, provides information concerning the preferred solution conformation of the peptide. When intact proteins are so labeled, isotopic monitoring of the probe by NMR or other means provides additional information concerning the tertiary structure of the molecules.

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#### REFERENCES

1. To whom inquires should be directed.
2. G. E. Means and R. E. Feeney, *Biochemistry*, 7, 2192-2201 (1968).
3. T. M. Joys and H. Kim, *Biochim. Biophys. Acta*, 581, 360-362 (1979).
4. L. Poncz and D. G. Dearborn, *J. Biol. Chem.*, 258, 1844-1850 (1983).
5. K. Fretheim, S. Iwai and R. E. Feeney, *Int. J. Peptide Protein Res.*, 14, 451-456 (1979).
6. E. M. Brown and R. Greenberg, *Fed. Proc.*, 42, 1763 (1983).
7. W. K. Paik, J. Z. Farooqui, T. Roy and S. Kim, *J. Chromatogr.*, 256, 331-334 (1983).
8. G. Goldstein, M. P. Scheid, E. A. Boyse, D. H. Schlesinger and J. Van Wauwe, *Science*, 204, 1309-1310 (1979).
9. J. J. Schmidt, S. Sathamoorthy, S. Rasmussen and B. R. DasGupta, *Fed. Proc.*, 42, 1811 (1983).

10. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.
  11. A. M. Weiner, T. Platt and K. Weber, *J. Biol. Chem.*, 247, 3242-3251 (1972).
  12. K. R. Woods and K-T. Wang, *Biochim. Biophys. Acta*, 133, 369-370 (1967).
  13. M. L. Groves and R. Greenberg, *Biochem. J.*, 169, 337-342 (1978).
  14. M. Henneske and B. V. Plapp, *Anal. Biochem.*, 136, 110-118 (1984).
  15. J. W. Marsh, A. Nahum and D. F. Steiner, *Int. J. Peptide Protein Res.*, 22, 39-49 (1983).
  16. D. Tourwe, J. L. DeCoen, K. Hallenga and G. Van Binst, *Int. J. Peptide Protein Res.*, 24, 84-93 (1984).
- IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, 243, 3557-3559 (1968).