GUANIDATION OF AMINO ACIDS AND PEPTIDES

by

Herbert Katsumi Iijima

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1964

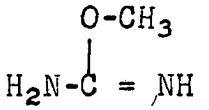
TABLE OF CONTENTS

I. INTRODUCTION

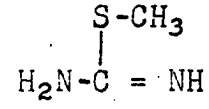
One of the methods of investigating protein structure and function has been the chemical modification of side groups such as amino, sulfhydryl, hydroxyl, and carboxyl functions. After such molecular alterations certain hormones and enzymes exhibit marked changes in biological activity. The use of formaldehyde for the inactivation of viruses in the preparation of vaccines is a well-known example of the alteration of activity through chemical modification of molecular structure.

Modification of proteins by converting amino groups to guanido groups has been carried out with various reagents, some of which are shown in Figure 1. Although most reagents used in protein modification attack more than one type of chemically reactive group, O-methylisourea has been shown to be specific for the amino group alone. The further specificity of this reagent for the epsilon-amino group of lysine has been proposed in a number of published papers (1, 2, 3, 4, 5). However, some investigators, using either O-methylisourea or 1-guanyl-3,5-dimethylpyrazole (GDMP), have shown the presence of alpha-guanido acids in protein hydrolysates (6, 7, 8, 9, 10, 11).

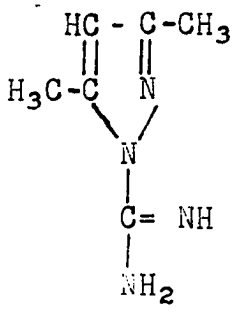
The reaction of the reagent with the epsilon-amino group results in the conversion of lysine to homoarginine; reaction with an alpha-amino acid produces an alpha-guanido acid. Reaction of O-methylisourea with an amino group is shown in



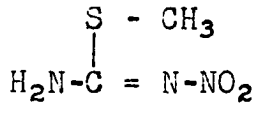
O-methylisourea



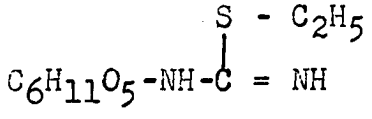
S-methylisothiourea



1-guanyl-3,5-dimethylpyrazole
(GDMP)



2-methyl-1(or 3)-
nitro-2-isothiourea
(MNT)



S-ethylglucosyl-
isothiourea
(SEG)

Figure 1. Some guanidating reagents

Figure 2. Synthesis of alpha-guanido acids by several groups of investigators has shown that alpha-amino acids are indeed attacked by a guanidating agent under mild conditions (12, 13, 14, 15, 16, 17). Since the unprotonated form of the amino group is the reactive species (Figure 2), an alpha-amino group with its lower pK would be expected to be more reactive than an epsilon-amino group at pH 8-10, where the reaction is usually carried out. There would thus seem to be no reason other than steric hindrance for lack of reaction between O-methylisourea and free terminal amino groups of proteins.

The objective of the present investigation was to determine the extent of reaction of alpha-amino as well as epsilon-amino groups of model compounds, amino acids and peptides, under the same mild conditions used for protein guanidation studies. Hydrolysis conditions were also chosen to conform to those used in protein structural determination.

Of the reagents used for guanidating proteins -- cyanamide, O-methylisourea, S-methylisothiourea, 1-guanyl-3,5-dimethylpyrazole (GDMP), 2-methyl-1(or 3)-nitro-2-isothiourea (MNT), S-ethylglucosylisothiourea (SEG), and its methyl analog (SMG) -- the last three (MNT, SEG, SMG have the disadvantage of introducing an additional moiety attached to the guanido group; the resulting bulky side chain could itself possibly inhibit biological activity of the protein.

Of the reagents which do not possess this disadvantage, cyanamide is least convenient for protein guanidation, and

$$R\text{-}NH_3^+ \rightleftharpoons R\text{-}NH_2 + H^+$$

$$CH_3O\text{-}C(NH_2)_2^+ \rightleftharpoons CH_3O\text{-}C(NH)NH_2 + H^+$$

$$R\text{-}NH_2 + CH_3O\text{-}C(NH)NH_2 \longrightarrow R\text{-}NH\text{-}C(NH)NH_2 + CH_3OH$$

$$R\text{-}NH\text{-}C(NH)NH'' + H^+ \rightleftharpoons R\text{-}NH\text{-}C(NH_2)_2^+$$

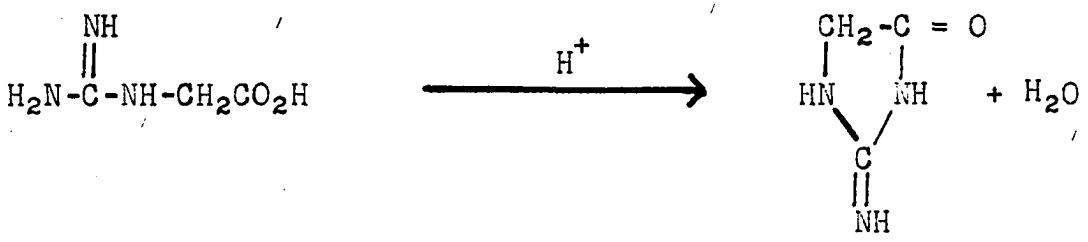
Figure 2. Reaction of O-methylisourea with an amino group

GDMP so new that relatively few studies have been carried out with it. S-methylisothiourea, although less expensive than its O-methyl analog, has the disadvantage of emitting the disagreeable odor of methyl mercaptan as the reaction proceeds. At the beginning of this investigation an efficient fume hood was not available and the author's fellow workers in the laboratory encouraged him not to use the S-methyl reagent. Furthermore, O-methylisourea was actually the reagent of choice anyway because it has been most widely used for protein guanidation studies.

This investigation was conceived primarily as an analytical study, on the assumption that failure to detect alpha-guanidation of proteins in the past may have been due to inadequate analytical technique rather than to specificity of the reaction. Even in instances where evidence has pointed to the reaction of N-terminal residues, indirect methods have been used to determine the presence of alpha-guanido compounds. These methods have made use of the ninhydrin, Sakaguchi, or Sanger DNFB (2,4-dinitrofluorobenzene) reagents. By using simple peptides instead of complex proteins as starting materials it was hoped that all products of the guanidation reaction could be adequately identified by column chromatography plus suitable colorimetric analyses.

The ninydrin reaction is positive for amino groups but negative for guanido groups. The Sakaguchi reaction is positive for mono-substituted but not di-substituted

guanidines; thus the positive Sakaguchi reaction of an alpha-guanido acid is lost as the acid undergoes cyclization to a glycocyamidine derivative. Such cyclization takes place under acid conditions as exemplified by the familiar creatine-creatinine conversion (Figure 3). After acid hydrolysis of a guanidated protein or peptide, therefore, it is necessary to look for the cyclic products in the hydrolysate; fortunately the Jaffé alkaline picrate reagent commonly used to detect creatinine gives an orange color with these other glycocyamidine derivatives also.



(Glycocyamine)

Ninhydrin -
Sakaguchi +
Jaffé -

(Glycocyamidine)

Ninhydrin -
Sakaguchi -
Jaffé +

Figure 3. Cyclization of an alpha-guanido acid

II. REVIEW OF LITERATURE

Interpretation of results of protein guanidation studies has been difficult because of uncertainty regarding the reactivity of terminal alpha-amino groups toward the guanidating agent. Some investigators have found that the terminal alpha-amino group is affected as well as the epsilon-amino of lysine (6, 7, 8, 9, 10, 11). Among the alpha-amino groups which have been guanidated are those of glycine, phenylalanine, aspartic acid, alanine, serine, threonine, leucine, and lysine. The proteins in which alpha-guanido products have been found after guanidation are insulin, serum albumin, beta-lactoglobulin, corticotropin A, and ribonuclease (subtilisin-modified, peptide fragment).

A. Presence of Alpha-Guanido Acid Residues in Guanidated Proteins

The first report of reaction of alpha-amino groups of proteins with O-methylisourea appeared in 1954 (6). In an abstract Saroff and Irreverre proposed the use of this reagent for the determination of N-terminal residues in proteins. They tentatively identified by electrophoresis and paper chromatography the guanido derivatives of alanine, serine, and threonine in a hydrolysate of guanidated bovine serum albumin.

The presence of guanido derivatives of glycine (50 percent of the N-terminal residues) and phenylalanine (10 percent of the N-terminal residues) in zinc insulin modified with the same reagent was reported by Evans and Saroff in 1957 (7). Using column chromatography these authors found that the alpha-guanidophenylpropionic acid peak completely disappeared with the appearance of a new peak positive to the acetylbenzoyl reagent (for guanidines) emerging with histidine. When glycocyamine and guanidophenylpropionic acid were added to the modified protein before hydrolysis, the guanidophenylpropionic acid peak again disappeared. The unknown acetylbenzoyl-positive component appeared with histidine in both column and paper chromatography. Complete cyclization of guanidophenylpropionic acid would account for these observations, no doubt, but the authors failed to mention possible cyclization of alpha-guanido acids under the acid conditions of protein hydrolysis.

Habeeb, in search of a milder and more stable reagent, studied the reaction of proteins with GDMP at pH 9.5 (8, 9). Only about 10 percent of this reagent was decomposed to 3,5-dimethylpyrazole and urea after 42 days at 0°C. In contrast, O-methylisourea decomposes at a rate of 41 percent per day at room temperature (18). Using the DNFB method, Habeeb was able to show that 60 percent of the N-terminal aspartic acid in GDMP-treated bovine serum albumin had been converted to the guanido derivative. By the same method he also showed

that 30 percent of the N-terminal leucine of beta-lactoglobulin had reacted with the reagent.

When ribonuclease is treated with subtilisin it is cleaved into two segments, a protein moiety (S-protein) and a 20-amino acid fragment (S-peptide). There are two lysine residues in the S-peptide, in positions 1 and 7. Guanidation of both epsilon-amino groups would result in a diguanido peptide; the additional reaction of the alpha amino of the first lysine would result in a triguanido derivative. Vithayathil and Richards, in studying the catalytically reactive groups of ribonuclease, found that one of their preparations contained a mixture of diguanido and triguanido derivatives of the S-peptide (10). Separation of these two products by electrophoresis was followed by column amino acid analysis. Since only the ninhydrin test was applied to effluent fractions, the presence of any diguanidated lysine could not be detected. Instead, evidence for the latter was gained through the failure of acetic anhydride to react with the triguanido S-peptide.

The S-peptide activity was determined by measuring hydrolysis of RNA and uridine-2',3'-phosphate by mixtures of various amounts of S-peptide and a fixed amount of S-protein. The acetylated S-peptide was shown to have a reduced activity, attributed to acetylation of essential epsilon-amino groups. Neither deamination of the alpha-amino of lysine-1 nor acetylation of the same group in the diguanido S-peptide

caused any loss of activity. Thus, catalytic activity associated with the S-peptide moiety of ribonuclease seems to be dependent upon the presence of a positive charge on lysine side chains.

Corticotropin (ACTH) and alpha-melanocyte-stimulating hormone (alpha-MSH) possess an identical tridecapeptide sequence. The former terminates in serine and the latter in N-acetylserine. Since corticotropin possesses only a small fraction (1 percent) of alpha-MSH activity, Ellington attempted to increase its activity in this regard by selective acetylation of the terminal serine residue (11). He had hoped to guanidate only the epsilon-amino groups of lysine first, leaving the amino of serine free for subsequent reaction with acetic anyhdride. By the use of electrophoresis and phenylthiohydantoin techniques, he found to his surprise that the N-terminal serine of corticotropin had completely disappeared upon treatment of this hormone with S-methylisothiourea. The reason given for the reaction of this residue was that "in large molecules of this kind, the alpha-amino residues may have pK values somewhat modified by their environment with other amino acids and therefore would behave altogether differently from 'normal' alpha-amino groups."

B. Guanidated Proteins in which no Alpha-Guanido Acids have been found

Roche *et al.* in studying the guanidation reaction with several proteins, found no evidence of alpha-guanidation (19). By paper chromatography it was determined that the epsilon-amino groups of the following proteins reacted quantitatively with either S-methylisothiourea of O-methylisourea: casein, hemoglobin, lysozyme, and ovalbumin. Some of the lysine residues of thyroglobin and gelatin were unreactive.

Guanidation of chymotrysinogen by Chervenka and Wilcox resulted in quantitative conversion of lysine to homoarginine residues (3). Evidence for lack of alpha-guanidation was the observation that the modified zymogen was still reactive to carbon disulfide. The activated guanidochymotrypsinogen exhibited enzymic activity similar to that of delta-chymotrypsin.

Klee and Richards, in 1957 guanidated ribonoclease and found no evidence for the reaction of the alpha-amino group of the N-terminal lysine (18). However, only 0.3 mole of alpha-DNP-homoarginine was recovered per mole of DNFB-treated guanidated enzyme, and the authors did not exclude the possibility of some alpha-guanidation. Ribonuclease guanidated to the extent of 95 percent retained 33 percent enzymic activity, while complete conversion of all epsilon-amino groups caused total inactivation. Modification of the last lysine residue was believed to cause a critical disturbance

of protein three-dimensional structure.

In determining the effect of guanidation on biologically active proteins Geschwind and Li modified lactogenic hormone, growth hormone, and lysozyme, and found no alteration of activity (2). Treatment with O-methylisourea resulted only in the conversion of lysine to homoarginine residues, with no N-terminal alpha-guanido derivatives detectable by paper chromatography of the DNFB-treated proteins. Together with guanidation, subsequent acetylation was performed to illustrate the point that intact alpha-amino groups are not essential for activity of the three proteins studied. However, acetylation of epsilon-amino groups of lysine almost completely inactivated the proteins. Therefore, it is apparent that a positive charge on the lysine side chain is necessary for maintenance of activity, whether this charge is due to an epsilon-amino or a guanido group.

Takahashi et al. investigating groups essential for cytochrome C activity, treated this protein with both acetic anyhdride and O-methylisourea (4, 20). Assuming the latter reagent to be specific for epsilon-amino groups, the authors were able to demonstrate that this protein could be modified to the extent of 80 percent without alteration of its electron transferring ability in the succinic oxidase system. The presence of cationic side groups, as in the proteins studied by Geschwind and Li, was required for activity since

acetylated cytochrome C was inactive as an electron transferring agent.

The effect of guanidation on the properties of mercuripapain was examined by Shields et al. (21). Upon treatment with O-methylisourea the enzyme remained fully active, exhibiting the same substrate specificity as native papain. In contrast to the rapid inactivation of papain by nitrous acid, the guanidated derivative was only slowly inactivated and retained about half its original activity after 24 hours. Trypsin was unable to hydrolyze either benzoyl-L-homoarginin-amide or peptide bonds of guanido-mercuripapain involving the carboxyl group of homoarginine.

Upon treatment of trypsin with S-methylglucosylisothiourea, Maekawa and Liener have converted three lysine epsilon-amino groups into glucosylamidyl groups without affecting the N-terminal isoleucine (5). However, it is believed by the authors that the reagent reacted with a ring nitrogen of all three of the histidine residues. The use of this reagent has been suggested in studying proteins whose biological activity might depend on unsubstituted histidine residues.

C. Summary

As seen in Table 1, nearly thirty different proteins have been guanidated, but only a few investigators have determined the effect of reaction on biological activity or on terminal alpha-amino groups. Many of the reports on guanidation have

Table 1. Summary of protein guanidation

Protein	Reagent[a]	pH	Percent guanidation	Alpha-guanido	Biol. act.	Ref.
1. Zinc insulin	OMI	10	90-100	Gly, 50% Phe, 10%	unchanged	7
Insulin	SEG	8	-	-	-	22
2. Corticotropin A_1	SMT	10.5	100	Ser, 100%	-	11
3. Beta-lactoglobulin	GDMP	9.5	97	Leu, 30%	-	8,9
4. Serum albumin						
a. Human	OMI	10.5-11	93-98	Possible	-	23
Human	OMI	9.5-10.5	-	Ala, Ser, Thr	-	6
Human	MNT	9.0	36	-	-	24
b. Horse	OMI	-	-	-	slight change	25
Horse	SEG	8-8.1	25-50	-	-	26
Horse	SEG	8	37	-	-	22
c. Bovine	OMI	9.5-10.5	-	Ala, Ser, Thr	-	6
Bovine	OMI	-	-	-	slight change	25

[a]OMI: O-methylisourea; SEG: S-ethylglucosylisothiourea; SMT: S-methylisothiourea; GDMP: 1-guanyl-3,5-dimethylpyrazole; MNT: 2-methyl-1(or 3)-nitro-2-isothiourea; SMG: S-methylglucosylisothiourea.

Table 1. (Continued)

Protein	Reagent[a]	pH	Percent guanidation	Alpha-guanido	Biol. act.	Ref.
4. Serum albumin (cont.)						
c. Bovine	GDMP	9.5-10	-	Asp,60%	-	8,9
Bovine	MNT	9.0	36	-	-	24
Bovine	SEG	8	50	-	-	22
5. RNase	OMI	8.5-11	95 100	No No	77% loss 100% loss	19
RNase	OMI	10.5-11	100	No	80% loss	2
S-peptide	OMI	10.0	100	Lys	unchanged	10
6. Alpha-chymotrypsinogen	OMI	10.3	100	No	-	3
7. Mercuripapain	OMI	10.2	88	No	unchanged	21
8. Growth hormone	OMI	10.5	91-96	No	unchanged	2
9. Lactogenic hormone	OMI	10.5-11	100	No	unchanged	2
10. Alpha-corticotropin	OMI	10.5-11	75	No	unchanged	2
11. Lysozyme	OMI	10.5-11	100	No	unchanged	2
Lysozyme	OMI or SMT	-	100	No	-	18

Table 1. (Continued)

Protein	Reagent[a]	pH	Percent guanidation	Alpha-guanido	Biol. act.	Ref.
12. Cytochrome C	OMI	10.9-11.0	80	No	unchanged	4
Cytochrome C	OMI	10.5-11	92	-	unchanged	27
13. Trypsin	SMG	8.4	23	No	slight loss	5
14. Serum globulin						
a. Bovine	SEG	8-8.1	-	-	-	26
b. Horse	SEG	8	39	-	-	22
15. Gelatin	OMI or SMT	-	-	No	-	19
Gelatin	OMI	10-12	Lys, 90 OH-Lys, 87	-	-	28
Gelatin	OMI	10-11	100	-	-	29
Gelatin	OMI	10.5	-	-	-	30
Gelatin	SEG	8-8.1	25-50	-	-	26
Gelatin	SEG	8	44	-	-	22
16. Ichthyocol	GDMP	9.5	Lys, 87 OH-Lys, 75	-	-	31
Ichthyocol gelatin	GDMP	9.5	Lys, 94 OH-Lys, 87	-	-	31

Table 1. (Continued)

Protein	Reagent[a]	pH	Percent guanidation	Alpha-guanido	Biol. act.	Ref.
17. Casein	SMT	-	20-30 (3d.) 84-90 (3 wk.)	-	-	14
Casein	SMT or OMI	-	100	-	-	19
Casein	SEG	8	52	-	-	22
Alpha-casein	SMT	-	100	-	-	32
Beta-casein	SMT	-	100	-	-	32
18. Lactalbumin	SMT	-	20-30 (3 d.) 85-90 (3 wk.)	-	-	14
Lactalbumin	OMI	10.5	100	-	-	33
19. Fibrinogen	OMI	10.5-11	75	-	-	34
20. Tropomyosin	OMI	10.5-11 11.0-11.5	20-41 94	-	-	35
Tropomyosin	SMT	9.0	91	-	-	36
21. TMV	OMI	10.5	Nil	-	-	37
(Denatured)	OMI	10.5	50	-	-	37
22. Collagen	OMI	-	65	-	-	38
23. Ovalbumin	OMI or SMT	-	100	-	-	19

Table 1. (Continued)

Protein	Reagent[a]	pH	Percent guanidation	Alpha-guanido	Biol. act.	Ref.
24. Hemoglobin	OMI or SMT	-	100	-	-	19
25. Thyroglobin	OMI or SMT	-	< 100	-	-	19
26. Edestin	SEG	8	43	-	-	22
27. Zein	SEG	8	Nil	-	-	22
28. Tyrocidine	SMT	-	Orn, 100	-	-	1

dealt with physical properties such as solubility, viscosity, sedimentation velocity, and electrophoretic behavior. Other investigators have used the guanidation reaction in order to determine N-terminal residues, to check the specificity of trypsin, and to determine the presence of epsilon peptide linkages in structural proteins.

III. EXPERIMENTAL MATERIALS AND METHODS

A. Materials

O-Methylisourea sulfate was obtained from Eastman Kodak Company and recrystallized from methanol-acetone before use in guanidation reactions.

Glycyl-L-lysine sulfate, L-lysylglycine sulfate, and glycocyamine were of chromatographically pure grade from Mann Research Laboratories.

Glycylglycine, glutathione (oxidized), L-lysine hydrochloride, DL-2,3-diaminopropionic acid hydrobromide and L-2,4-diaminobutyric acid dihydrochloride were all obtained from Nutritional Biochemicals Corporation.

Chromatographically pure L-homoarginine sulfate was prepared according to the method of Kurtz (39). From 15 grams of lysine hydrochloride, 18.4 grams of cupric carbonate, and 20 grams of O-methylisourea sulfate, was obtained a low yield of product which had a nitrogen content of 19.80 percent (theoretical: 19.57 percent) and softened at 180°C. (reported: 183-188°C.) after drying in vacuo at 100°C. overnight. The product obtained was found to be chromatographically pure as determined by analysis on the 0.9 x 15 cm. column of Amberlite IR-120 described below.

Cyclized alpha-guanido-epsilon-aminocaproic acid dihydrochloride was prepared according to the method of Steib

(40). From five grams of DL-epsilon-benzoyllysine (Calbiochem) and 6.88 grams of O-methylisourea sulfate was obtained one gram of product which melted at 195°C. Analysis on the 0.9 x 15 cm. column revealed a single ninhydrin- and Jaffe-positive peak emerging with the alkaline effluent.

Resin for the 0.9 x 15 cm. analytical column was purchased from Bio-Rad Laboratories, catalog designation Aminex MS, Fraction C, 33-47 microns. This resin is referred to as Amberlite IR-120 in this dissertation. The AG 50W-X8 (200-400 mesh) resin was also purchased from Bio-Rad Laboratories. This resin is sometimes referred to as Dowex 50 in this dissertation.

Amberlite IRC-50 (100-200 mesh) was obtained from Rohm and Haas Company.

B. Guanidation Method

The guanidation procedure followed in this investigation was adopted from that used by Hughes et al. (23). A typical run may be described as follows: To a cold solution containing O-methylisourea sulfate a hot, saturated solution of barium hydroxide was added dropwise until the alkalinity of the solution reached pH 8. After centrifugation and repeated washing of the precipitated barium sulfate, the amino acid or peptide was added to the clear supernatant. The reaction mixture was adjusted to pH 10-11 with 6 N sodium hydroxide and then diluted to volume. The concentration of the reagent at

0.5 M was twice that required for conversion of all available amino groups to guanido groups. The reaction mixture was placed in the cold room at 3°C. and the pH adjusted daily by dropwise addition of dilute sodium hydroxide.

When the peptide was in the form of its sulfate, it was dissolved along with the O-methylisourea, and the sulfate from both peptides and reagent was then removed simultaneously.

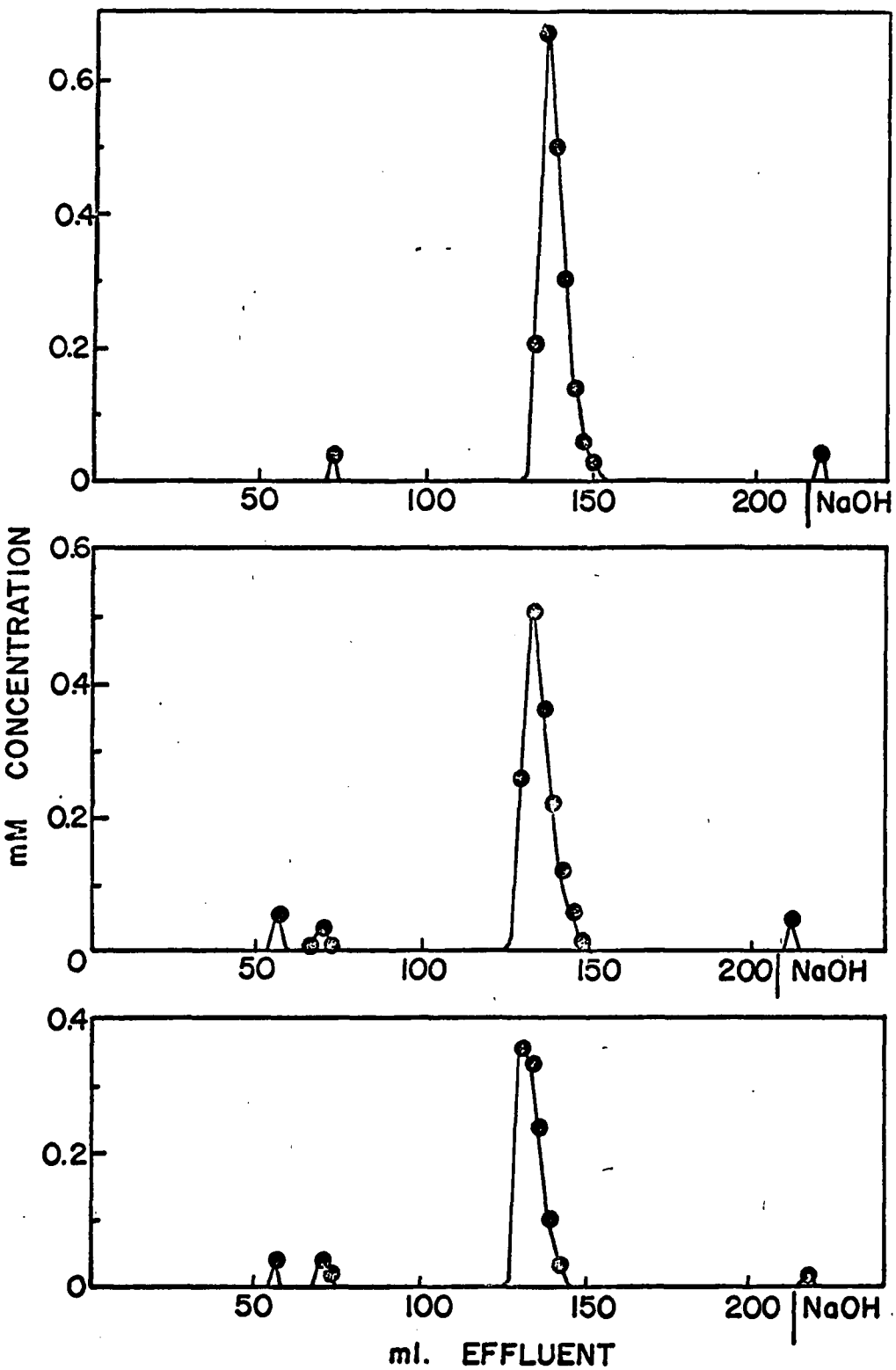
Since it was desirable to ascertain the rate of destruction of the guanidating agent under present reaction conditions, a solution containing only O-methylisourea was analyzed chromatographically after various periods of time. The sulfate was removed from a solution containing 0.4304 gram (2.5 millimoles) of O-methylisourea sulfate and the solution diluted to five ml. The solution at pH 10-11 was then stored in the cold room at 3°C. Examination of Figures 4 and 5 indicate that the reagent disappeared at a rate of 3.8 percent per day under the stated conditions. Extrapolation of the straight line of Figure 5 shows that the guanidating reagent was almost completely gone after 26 days. However, even after 10 days there would still be more than twice the amount necessary to react with the alpha-amino group of the preformed homoarginine in a lysine reaction mixture.

Figure 4. Hydrolysis of O-methylisourea at pH 10-11 and 3°C. in a 0.5 M solution
Column: 0.9 x 15 cm. Amberlite IR-120

Top: Amount present after 5 days
Middle: Amount present after 8 days
Bottom: Amount present after 22 days

The same volume of solution was withdrawn for each analysis.

● Ninhydrin


0.6
0.4
0.2
0
50
100
150
200
NaOH
mM CONCENTRATION
0.6
0.4
0.2
0
50
100
150
200
NaOH
0.4
0.2
0
50
100
150
200
NaOH
ml. EFFLUENT

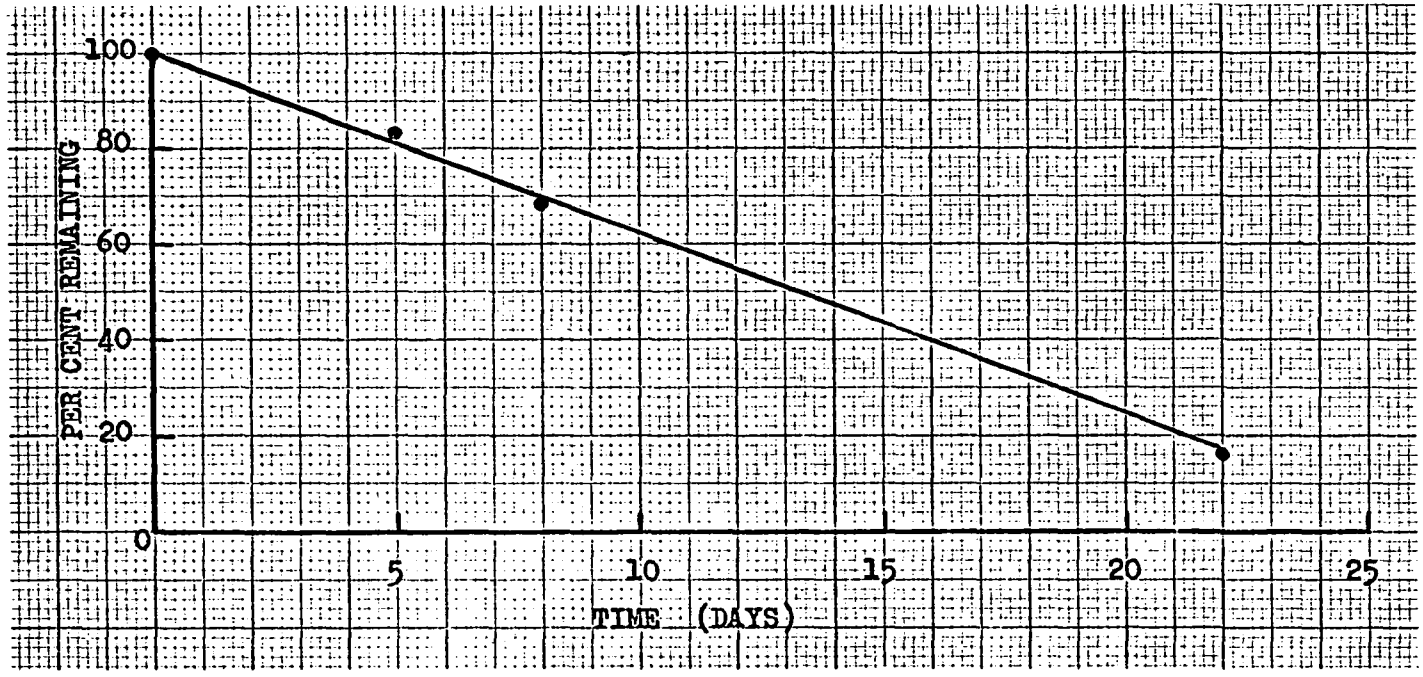


Figure 5. Hydrolysis of O-methylisourea at pH 10-11 and 3°C. in a 0.5 M Solution

C. Analytical Methods

Reaction mixtures or their hydrolysates were frationated by ion exchange chromatography for complete analysis of reaction products. Typically, a 0.9 x 15 cm. analytical column of Amberlite IR-120 was prepared and operated according to the procedure of Moore et al. (41). The pH 5.28 citrate buffer, with caprylic acid as preservative, was used for nearly the entire run, collecting one-ml. fractions. The final alkaline washes were also collected in one-ml. fractions and neutralized with hydrochloric acid before being subjected to colorimetric analysis. Alternate fractions were subjected to ninhydrin (42), Sakaguchi (43), and Jaffé tests (44) in order to locate material in the effluent. After development of color, readings were taken with a Klett colorimeter and converted to millimolar concentration (micromoles/ml.). Standard curves for the three colorimetric reactions were plotted using leucine, arginine, and creatinine respectively. When comparison samples were not available, 100 percent color yields were assumed.

Ninhydrin reagent was delivered into tubes containing the fractions by means of an automatic syringe instead of a pipetting machine connected to the reservoir (43). A pipetting machine (from Baltimore Biological Laboratory) was used to deliver 5 ml. of ethanol diluent from a storage bottle. A number 59 filter was used in taking colorimetric readings.

Leucine equivalents were obtained by reference to a leucine standard curve. Multiplying leucine equivalents by the color yield of the amino acid in question gave the concentration of that amino acid in micromoles/ml.

Various concentrations of several substances were prepared in pH 5.28 buffer and one-ml. samples were subjected to the ninhydrin reaction. Ninhydrin color yields relative to leucine were calculated as by Moore and Stein (42).

Ninhydrin Color Yields	
Glycyllysine	1.63
Lysylglycine	1.04
Glycylglycine	0.97
O-methylisourea	0.28
Creatinine	0.03
Glycocyamine	0.00

Sakaguchi analyses were run on ten tubes at a time, using two drops of hypobromite solution for each sample. After the addition of urea, five ml. of water was added to prevent condensation of moisture on the outer surface of the cuvette. Readings were taken with a number 52 filter in the colorimeter. A standard curve was obtained by using arginine in concentrations of 0.05-1.00 micromole/ml.

Solutions of various concentrations were prepared in pH 5.28 buffer and one-ml. samples run through the Sakaguchi

colorimetric reaction. Color yields of the three substances listed below were calculated relative to arginine color.

Sakaguchi Color Yields	
Homoarginine	1.00
Glycocyamine	0.55
O-methylisourea	0.00

Slight deviations from the method of Folin and Wu were made when performing the Jaffé test. To each of ten one-ml. samples were added 2.5 ml. of alkaline picrate reagent and 5 ml. of water. Color values were read with a number 52 filter 15 minutes after the addition of water. Concentrations were obtained from a standard curve of creatinine in concentrations of 0.05-1.00 micromole/ml.

For hydrolysis of reaction products, a calculated amount of the reaction mixture was neutralized and taken to dryness in a vacuum desiccator. The dried residue was dissolved in one ml. of constant-boiling hydrochloric acid and transferred to a hydrolysis tube. The mixture was frozen in a dry-ice and acetone bath, and the tube evacuated with a rotary oil pump and sealed with a gas-oxygen torch. After remaining in an oven at 120° C. for fifteen hours, the hydrolysate was then transfered to a five-ml. beaker and evaporated to dryness over sodium hydroxide pellets in a vacuum desiccator. The residue was dissolved in about 0.5 ml. water and evaporated to dryness again before being subjected to chromatographic

analysis.

D. Isolation of Products on a Preparative Scale

A 3.5 x 30 cm. jacketed column of AG 50W-X8(200-400 mesh) was employed for preparative scale isolations, the effluent being collected in 30-ml. fractions. This column also was operated with the pH 5.28 citrate buffer as eluant at a temperature of 50°C. A one-ml. aliquot was taken from each tube for colorimetric analysis. Separation of products on this column was comparable to that on the analytical column in each case where both were run. Figure 6 shows the fractionation of a standard mixture.

E. Desalting of Components

Dowex 50

Desalting with this resin followed the procedure of Drezé et al. (45). A 3.5 x 17 cm. column of AG 50W-X8(200-400 mesh) in the hydrogen phase was first loaded with a sample adjusted to pH 4. After 100 ml. of deionized water was run through, elution of sodium was begun with 0.5 N hydrochloric acid. Complete removal of sodium required about one liter of the acid. The subsequent 0.5 N and 4 N acid effluents were collected in 25-ml. fractions; colorimetric tests were done on neutralized one-ml. aliquots of these fractions. With this method, samples up to a volume of 2500 ml. could be desalted satisfactorily.

Figure 6. Separation of standards on preparative column 3.5 x 30 cm. AG 50W-X8

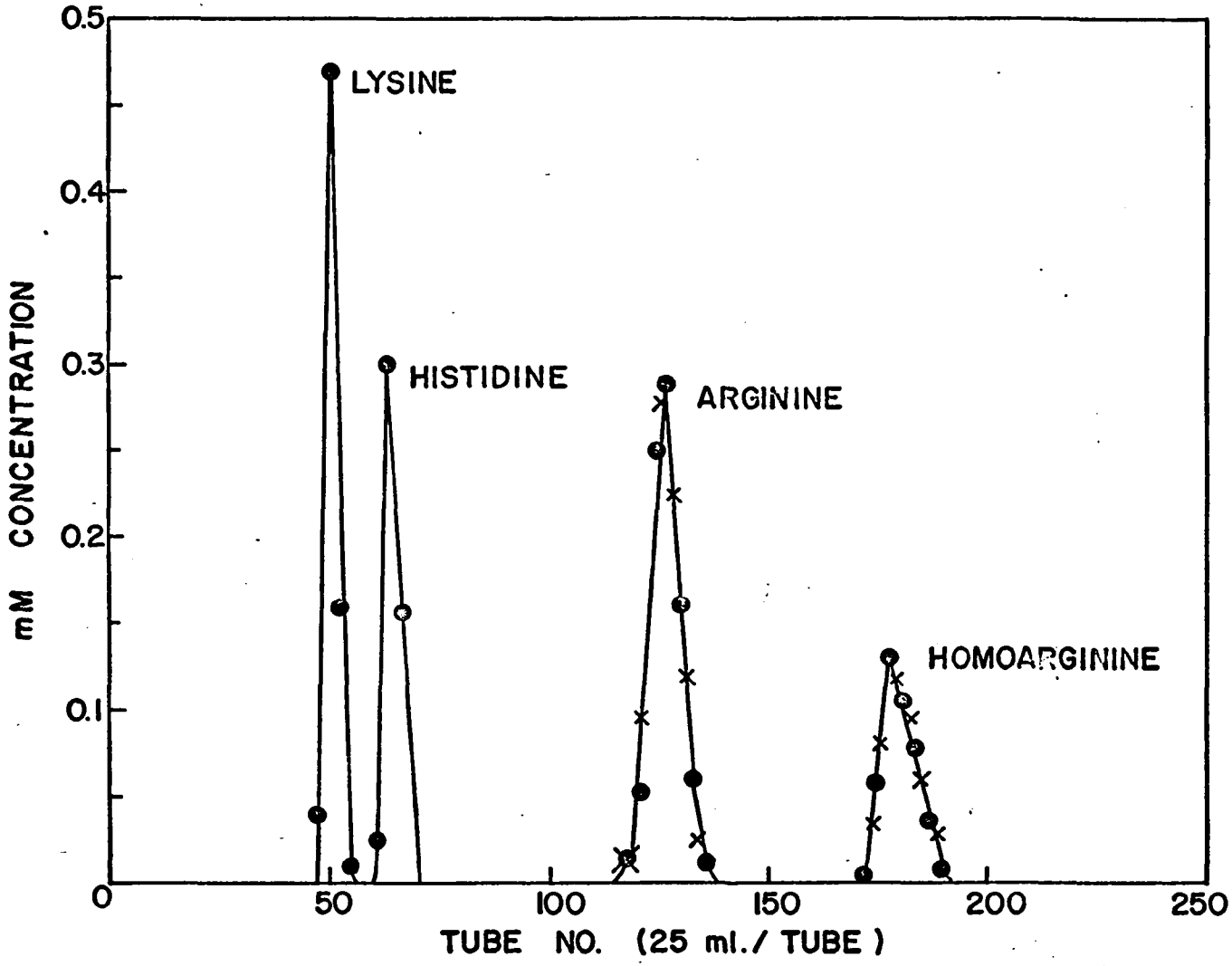
Load:

Arginine: 60 micromoles

Lysine, Histidine, Homoarginine: 30 micromoles

● Ninhydrin

x Sakaguchi


0.5
0.4
0.3
0.2
0.1
0
mM CONCENTRATION
LYSINE
HISTIDINE
ARGININE
HOMOARGININE
50
100
150
200
250
TUBE NO. (25 ml./ TUBE)

Amberlite IRC-50

Desalting of guanidated peptides necessitated use of a mild procedure in order to avoid peptide cleavage and cyclization of alpha-guanidated residues. For this purpose a modification of the method used by Winters and Kunin was employed (46). A 4.5 x 24 cm. column of Amberlite IRC-50 (100-200 mesh) in the hydrogen phase was first equilibrated with pH 5.28 citrate buffer. The sample at the same pH was then adsorbed and washed with a liter of deionized water. Complete removal of sodium required 500 to 1000 ml. of 0.1 N acetic acid, depending on the volume of the sample being desalted. Absence of sodium in the effluent could be determined by a negative zinc uranyl acetate reaction and by the fact that the pH of the effluent dropped from 7 to 3. Some components could be eluted with 0.1 N acetic acid, others requiring 1.0 N acetic acid. The largest volume of sample desalted using this system was about two liters.

IV. RESULTS

A. Lysine

Reactants:

O-methylisourea sulfate	4.304 gm. (25 millimoles)
L-lysine hydrochloride	1.141 gm. (6.25 milimoles)

The presence of sulfate did not seem to affect the product of lysine guanidations, so in this case sulfate was not removed with barium hydroxide in the preparative run. Instead, the pH was adjusted by the addition of 6 N sodium hydroxide to the cooled solution containing lysine and O-methylisourea. The solution was then diluted to 50 ml., making the concentration of the reagent 0.5 M and that of lysine 0.125 M.

After eight days in the cold room a sample containing 3.13 micromoles of amino acid (calculated according to the amount of lysine originally present) was analyzed on the 0.9 x 15 cm. column (Figure 7). Another portion of the reaction mixture was neutralized, evaporated to dryness, and submitted to acid hydrolysis conditions. The reaction mixture itself showed a single peak, both ninhydrin- and Sakaguchi-positive, containing O-methylisourea and homoarginine. The acid-treated sample showed a single large Sakaguchi peak containing homoarginine. A very large ninhydrin peak at 90 ml. contained ammonia resulting from hydrolysis of the guanidating reagent.

Figure 7. Guanidation of lysine

Column: 0.9 x 15 cm. Amberlite IR-120

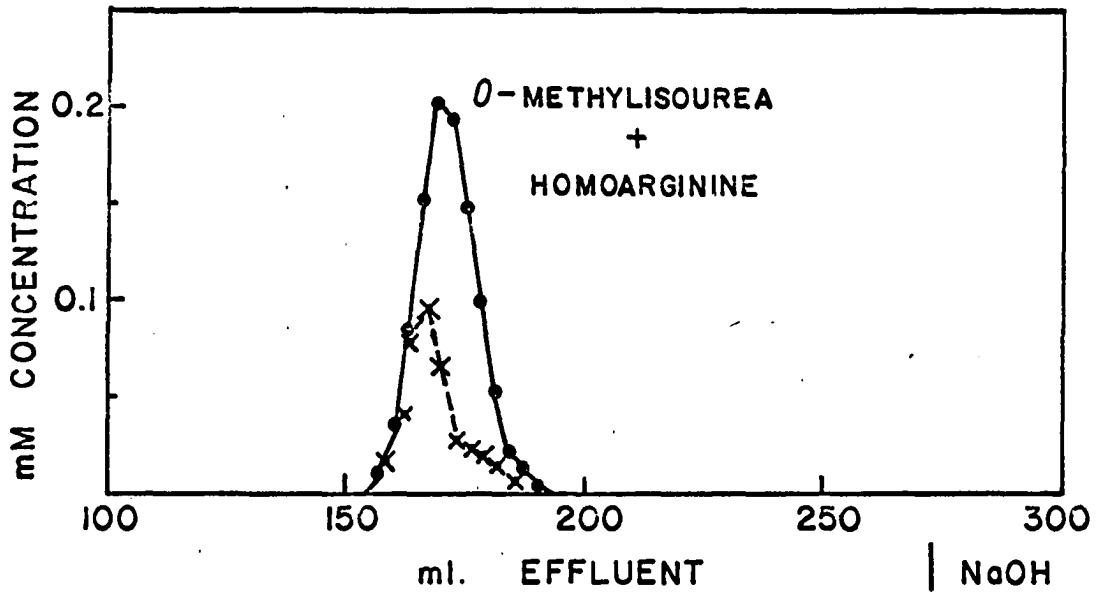
Top: Eight-day lysine reaction mixture

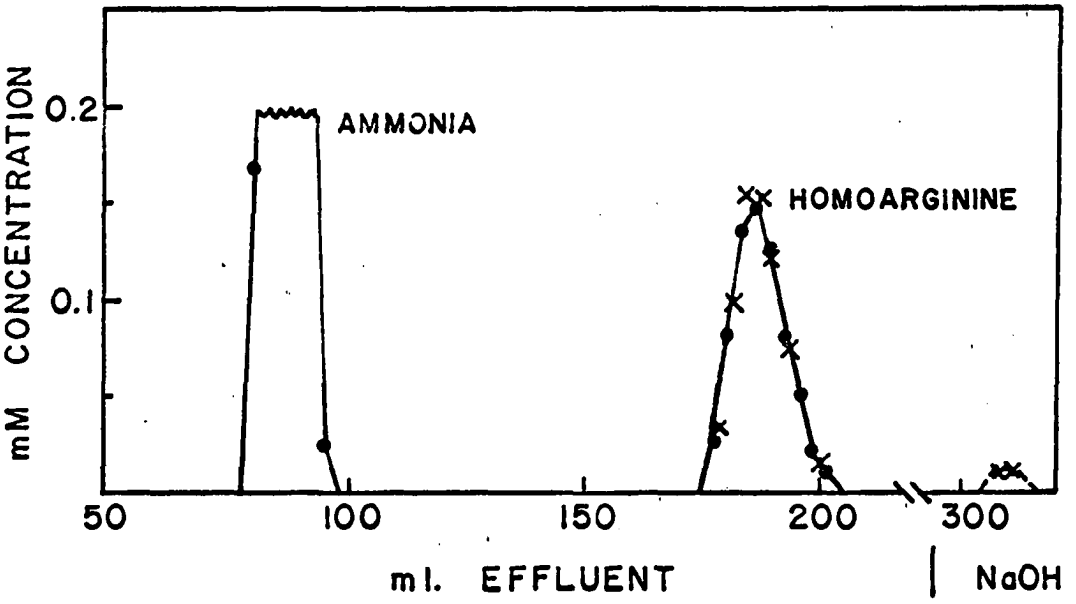
Bottom: Hydrolysate of reaction mixture

100 percent reaction of epsilon-amino group

● Ninhydrin

x Sakaguchi


mM CONCENTRATION
0.2
0.1
O-METHYLISOUREA
+
HOMOARGININE
100
150
200
250
300
ml. EFFLUENT
NaOH


mM CONCENTRATION
0.2
0.1
AMMONIA
HOMOARGININE
50
100
150
200
300
ml. EFFLUENT
NaOH

After samples were taken for analysis on the eighth day, the remainder of the reaction mixture was stored in a freezer. After three weeks, a 25 ml. sample was adjusted to pH 2.0-2.5 before being adsorbed on the preparative (3.5 x 30 cm.) column. It can be seen from Figure 8 that several other Sakaguchi-positive peaks had appeared by this time.

The fractions in peak 5 of the preparative column were pooled and desalted with the 3.5 x 17 cm. column of Dowex 50. The Sakaguchi-positive 4 N hydrochloric acid fractions from this desalting column were then evaporated to a syrup with a flash evaporator. The syrupy material was converted to its dipicrate salt and dried at 80°C. *in vacuo* overnight. The dipicrate of cyclized diguanidated lysine decomposed at 230°C. and had the following elemental composition:

$C_{20}H_{22}N_{12}O_{15}$ (MW 670.5)

Theoretical: 35.82% C, 3.31% H, 25.07% N

Found: 36.41% C, 3.38% H, 24.64% N

Desalting of the center portion of peak 2 was carried out in similar fashion. Ninhydrin-positive fractions from the desalting operation were combined and evaporated to dryness. The dry residue was crystallized twice from methanol-acetone. White needles of O-methylisourea hydrochloride were obtained which melted at 117°C. and had the following composition:

Figure 8. Guanidation of lysine. Fractionation of reaction mixture after remaining in cold room eight days and in freezer three weeks

Column: 3.5 x 30 cm. AG 50W-X8

1: Ammonia

2: O-methylisourea

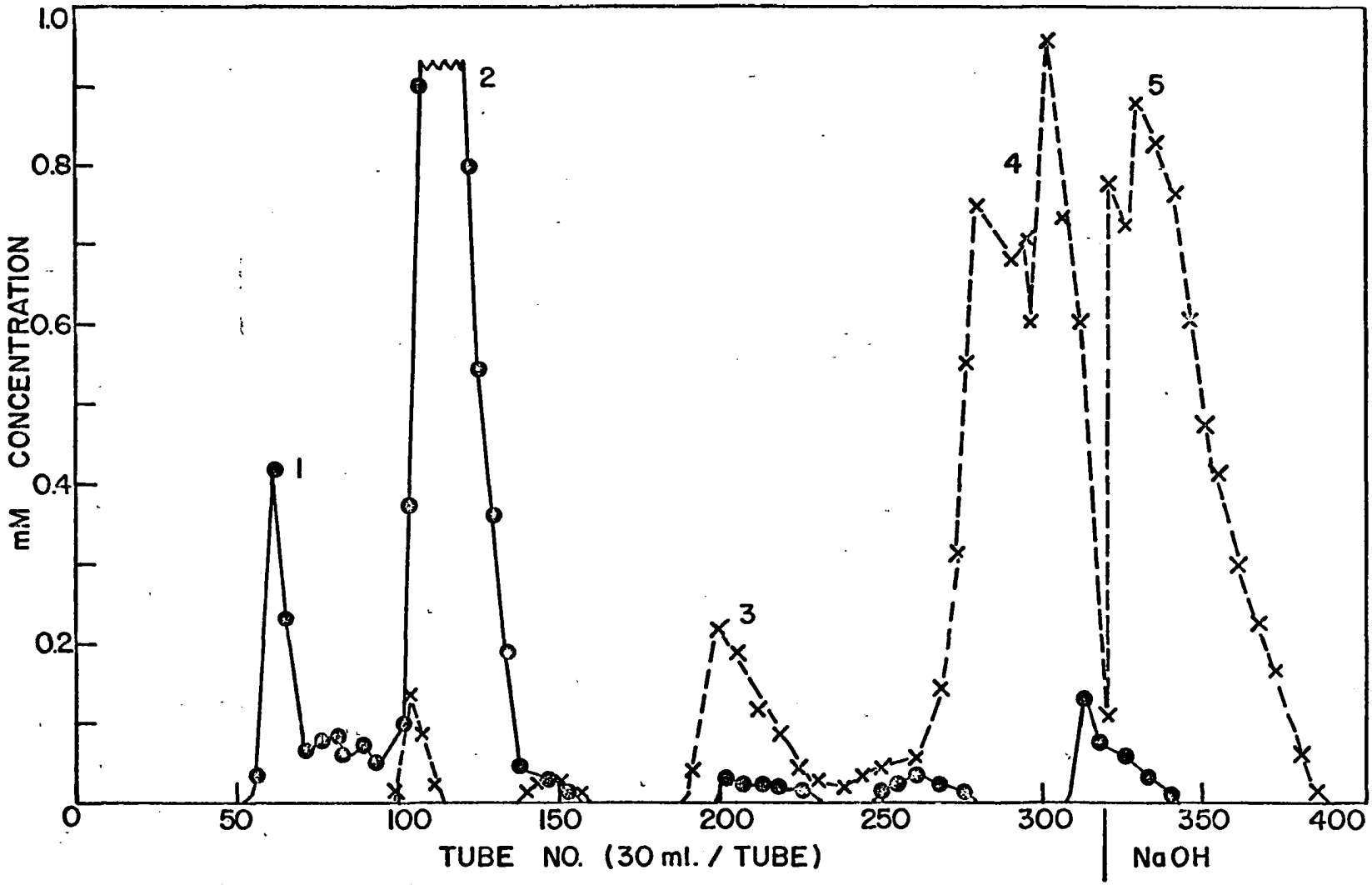
3: Unknown

4: Diguanidated lysine

5: Cyclized diguanidated lysine

● Ninhydrin

x Sakaguchi


1.0
0.8
0.6
0.4
0.2
0
mM CONCENTRATION
1
2
3
4
5
50
100
150
200
250
300
350
400
TUBE NO. (30 ml. / TUBE)
NaOH

$C_2H_7N_2OCl$ (MW 110.6)

Theoretical: 21.76% C, 6.39% H, 25.43% N

Found: 21.68% C, 6.30% H, 25.55% N

B. Arginine

Reactants:

O-methylisourea sulfate 2.152 gm. (12.5 millimoles)

L-arginine hydrochloride 1.317 gm. (6.25 millimoles)

Sulfate was removed from the reagent with barium hydroxide. The final volume was adjusted to 25 ml., making the solution 0.5 M in O-methylisourea and 0.25 M in arginine.

After remaining in the cold at pH 10-11 for six days, two 0.5-ml. aliquots were removed from the flask. One was chromatographed immediately, the other subjected to acid treatment. The presence of a large Sakaguchi-positive, ninhydrin-negative peak on the chromatogram of the reaction mixture indicated that the alpha-amino group had reacted (Figure 9). The hydrolysate contained a Jaffé-positive component emerging in the alkaline fractions probably corresponding to the cyclized alpha-guanidated arginine (Figure 9).

The component in the combined fractions of peak 3 from the preparative column (Figure 10) was desalted with the 4.5 x 24 cm. column of Amberlite IRC-50 described in the section on methods. The diguanido derivative of ornithine in the salt-free syrup was converted to its diflavinate salt

Figure 9. Guanidation of arginine

Column: 0.9 x 15 cm. Amberlite IR-120

Top: Arginine reaction mixture, six day sample

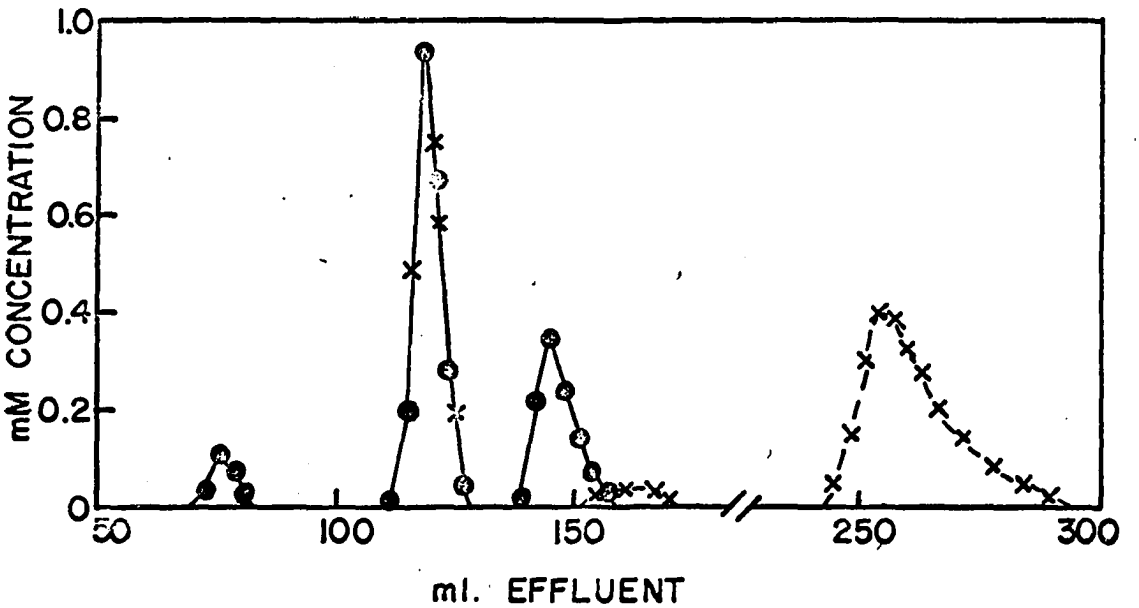
Bottom: Hydrolysate of six-day reaction mixture

51 percent reaction of alpha-amino group

● Ninhydrin

x Sakaguchi

Δ Jaffé


1.0
0.8
0.6
0.4
0.2
0
mM CONCENTRATION
50
100
150
250
300
ml. EFFLUENT

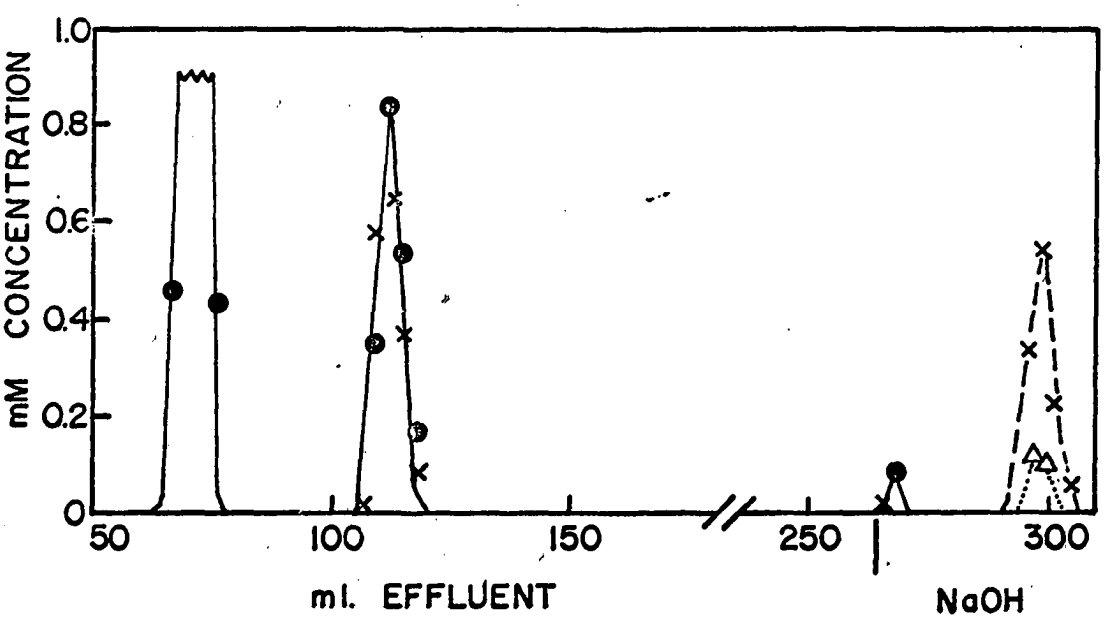

1.0
0.8
0.6
0.4
0.2
0
mM CONCENTRATION
50
100
150
250
300
ml. EFFLUENT
NaOH

Figure 10. Guanidation of arginine. Six-day reaction mixture

Column: 3.5 x 30 cm. AG 50W-X8

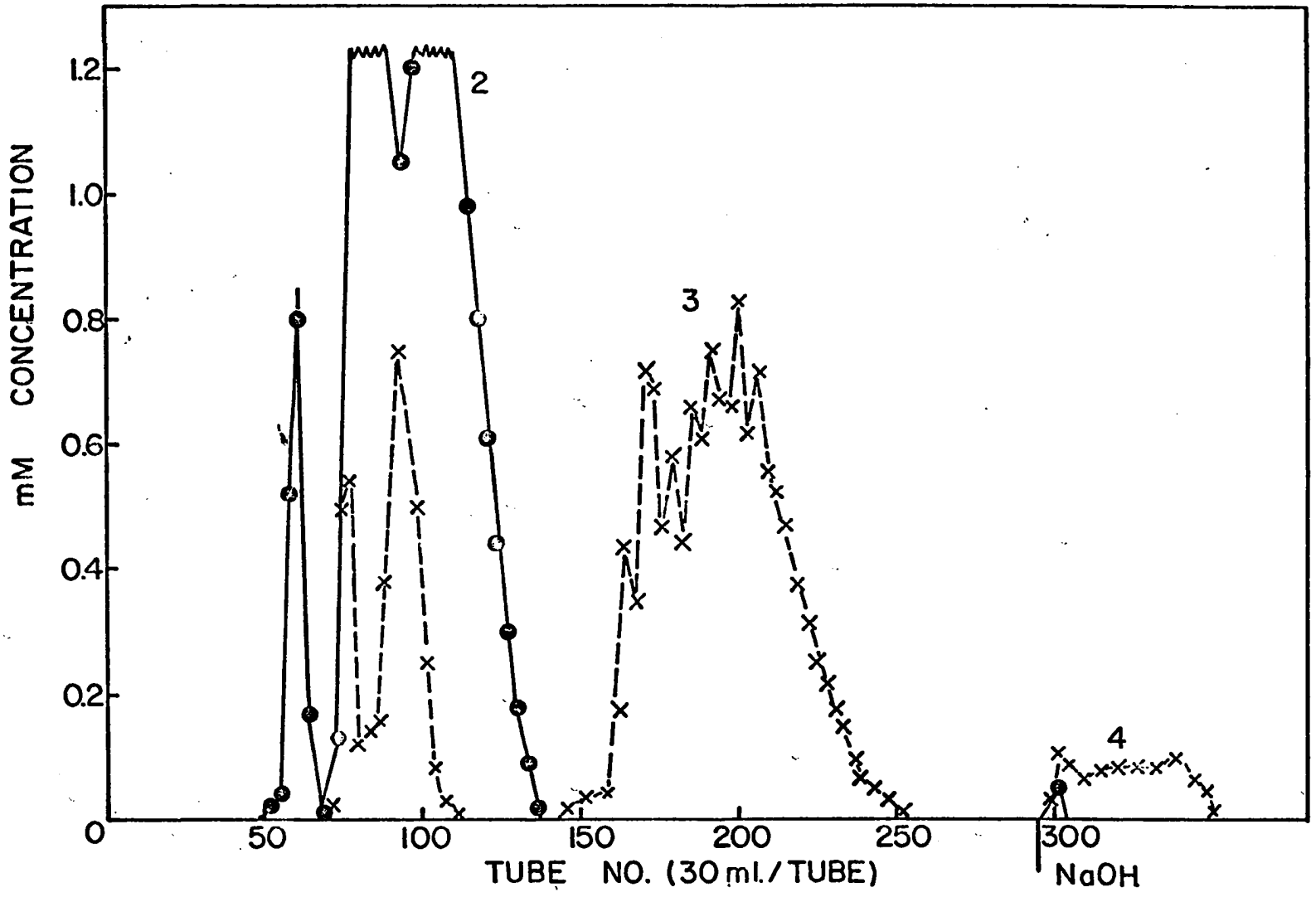
1: Ammonia

2: O-methylisourea and arginine

3: Diguanido-ornithine

● Ninhydrin

x Sakaguchi


mM CONCENTRATION
1.2
1.0
0.8
0.6
0.4
0.2
0
2
3
4
50
100
150
200
250
300
TUBE NO. (30 ml./TUBE)
NaOH

by addition of a saturated solution of flavianic acid to the filtered solution of the syrup. This diflavianate of the product resulting from guanidation of the alpha-amino group of arginine decomposed at 295°C. and had the following elemental composition:

$C_{27}H_{28}N_{10}O_{18}S_2$ (MW 444.8)

Theoretical: 36.39% C, 3.35% H, 16.58% N

Found: 36.98% C, 3.44% H, 16.43% N

C. Diaminopropionic Acid (DAPA)

Reactants:

O-methylisourea sulfate	0.8609 gm. (5 millimoles)
DL-2,3-DAPA·HBr	0.2312 gm. (1.25 millimoles)

The sulfate-free reaction mixture was diluted to 10 ml., giving a solution 0.5 M in O-methylisourea and 0.125 M in DAPA.

Examination of Figure 11 reveals only one major Sakaguchi peak, presumably alpha-amino-beta-guanidopropionic acid. The small Sakaguchi peak emerging with O-methylisourea probably contained the diguanido derivative.

In contrast to the results obtained with arginine, only a slight reaction of the alpha-amino group of diaminopropionic acid occurred.

Figure 11. Guanidation of diaminopropionic acid

Column: 0.9 x 15 cm. Amberlite IR-120

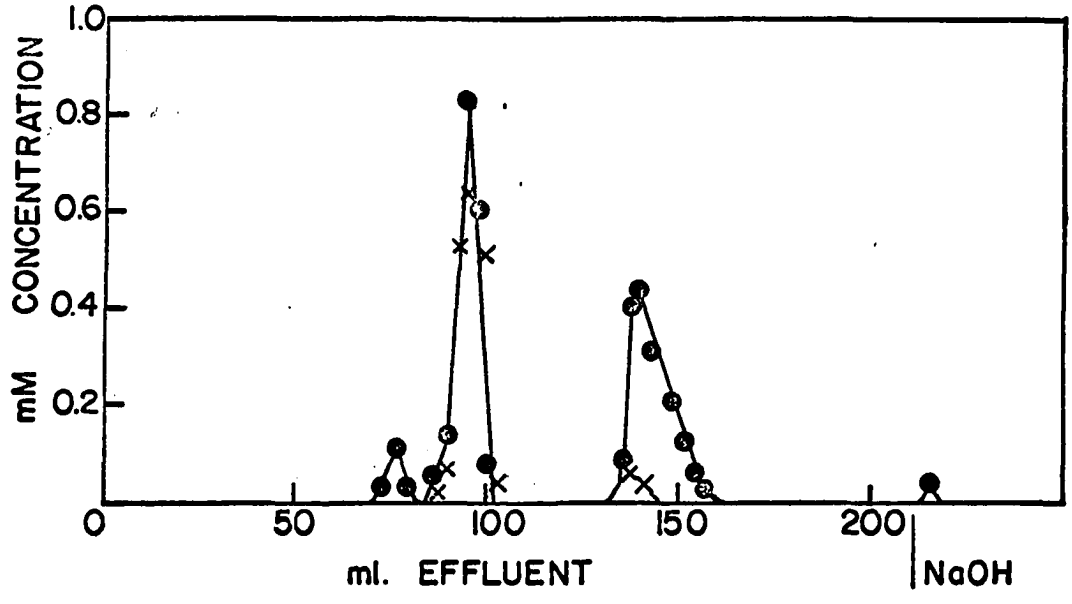
Top: Two-day sample

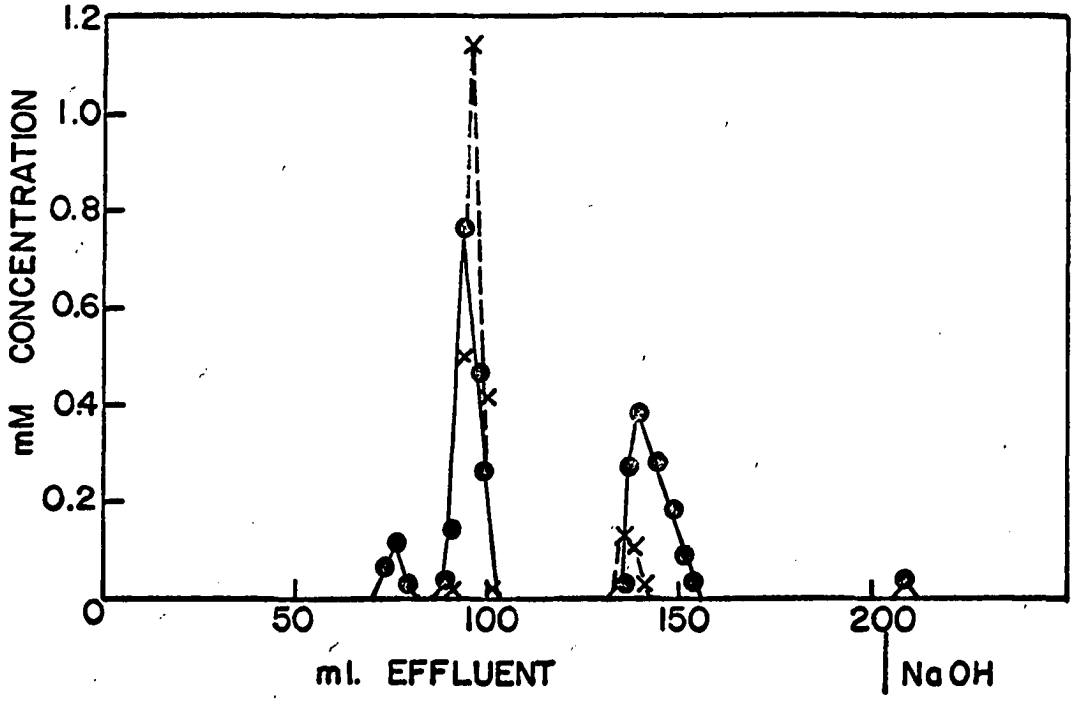
Bottom: Twelve-day sample

100 percent reaction of the beta-amino group within two days

● Ninhydrin

x Sakaguchi


1.0
0.8
0.6
0.4
0.2
mM CONCENTRATION
0
50
100
150
200
ml. EFFLUENT
NaOH


1.2
1.0
0.8
0.6
0.4
0.2
mM CONCENTRATION
0
50
100
150
200
ml. EFFLUENT
NaOH

D. Diaminobutyric Acid (DABA)

Reactants:

O-methylisourea sulfate	0.8609 gm. (5 millimoles)
L-2,4-DABA·2HCl	0.2387 gm. (1.25 millimoles)

The sulfate-free reaction mixture was prepared in the usual manner, adjusted to pH 10-11 and diluted to 10 ml. Final concentrations: 0.5 M O-methylisourea; 0.125 M DABA.

Like diaminopropionic acid, diaminobutyric acid appears to yield only a slight amount of the diguanido derivative (Figure 12).

E. Glycylglycine

Reactants:

O-methylisourea sulfate	0.860 gm. (5 millimoles)
Glycylglycine	0.330 gm. (2.5 millimoles)

After the usual procedure of sulfate removal, the mixture was adjusted to pH 10-11 and diluted to 10 ml. to give a solution 0.5 M in O-methylisourea and 0.25 M in glycylglycine. A precipitate was observed at the bottom of the flask after it had stood in the cold room about three hours. Centrifugation gave a recovery of 170 mg. of precipitate.

Samples of the precipitate were hydrolyzed for separation on both analytical and preparative columns. During the early stage of this work the resin used in the preparative runs was

Figure 12. Guanidation of diaminobutyric acid

Column: 0.9 x 15 cm. Amberlite IR-120

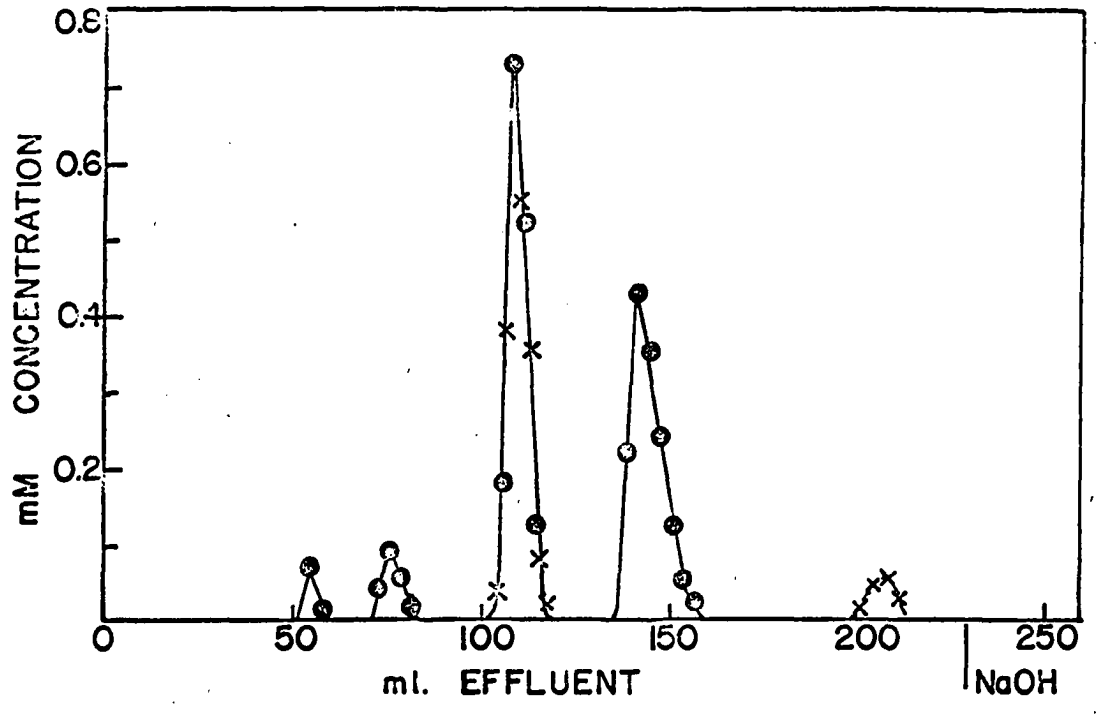
Top: Four-day sample

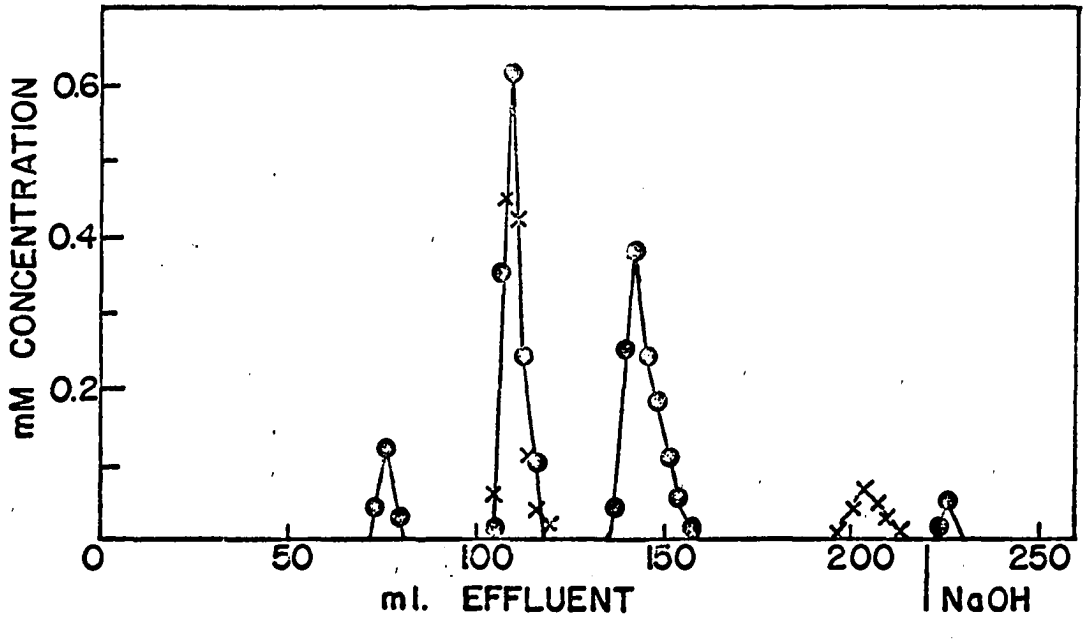
Bottom: Nine-day sample

100 percent reaction of gamma-amino group within nine days

● Ninhydrin

x Sakaguchi


0.8
0.6
0.4
0.2
mM CONCENTRATION
0
50
100
150
200
250
ml. EFFLUENT
NaOH


0.6
0.4
0.2
mM CONCENTRATION
0
50
100
150
200
250
ml. EFFLUENT
NaOH

the dark form of Dowex 50 (200-400 mesh) from Microchemical Specialties Company. Since this resin gave an elution pattern (Figure 13) different from that of the analytical column in this case its use was soon abandoned. The analytical column gave the chromatogram seen in Figure 14.

F. Glycyllysine

Reactants:

O-methylisourea sulfate	2.152 gm. (12.5 millimoles)
Glycyl-L-lysine sulfate	0.940 gm. (3.12 millimoles)

After removal of sulfate from the solution containing the reactants, the solution was adjusted to pH 10-11 with 6 N sodium hydroxide and diluted to 25 ml. Concentrations of O-methylisourea and glycyllysine were 0.5 M and 0.125 M, respectively.

Separation of products on a preparative scale resulted in the appearance of about four Sakaguchi-positive peaks (Figure 15).

Analysis of some of the desalted and hydrolyzed components indicated that peak 3 was glycylhomoarginine and peak 5, glycocyaminylhomoarginine (Figure 16). Peak 6 is probably the cyclized derivative of the latter substance. The identity of the component in peak 4 is uncertain.

Figure 13. Guanidation of glycylglycine. Hydrolysate of 100 mg. precipitate

Column: 3 x 35 cm. Dowex 50 (MISCO)

1: Glycine

2: Glycocyamine

3: Ammonia

4: Glycocyamidine

● Ninhydrin

x Sakaguchi

Δ Jaffé

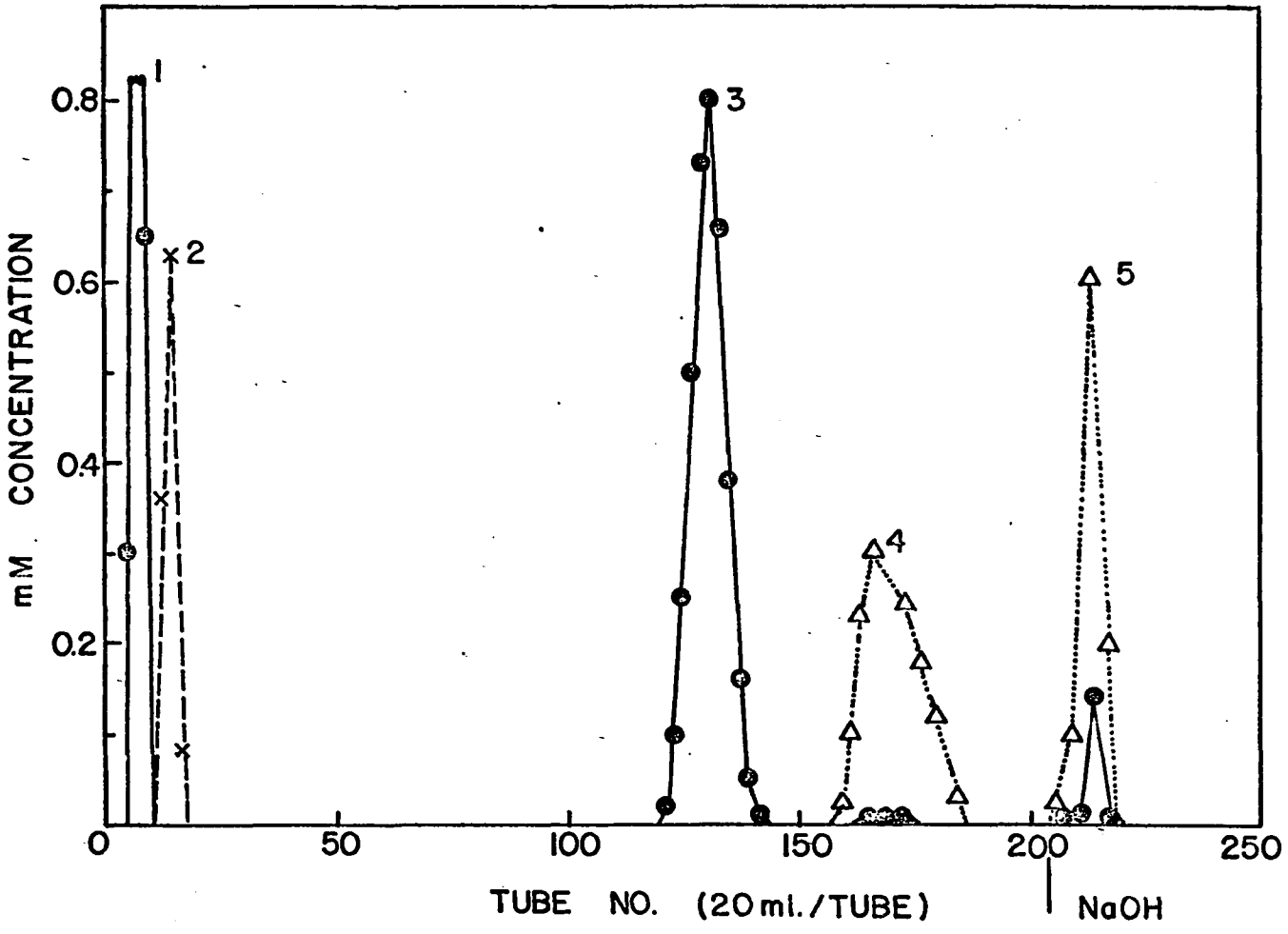

mM CONCENTRATION
0.8
0.6
0.4
0.2
0
50
100
150
200
250
TUBE NO. (20 ml./TUBE)
NaOH
1
2
3
4
5

Figure 14. Guanidation of glycylglycine

Column: 0.9 x 15 cm. Amberlite IR-120

Top: Analysis of hydrolysate

1: Glycine

2: Glycocyamine

3: Glycocyamidine

4: Ammonia

Bottom: Further acid treatment of peak 4 from preparative volumn

1: Glycocyamine

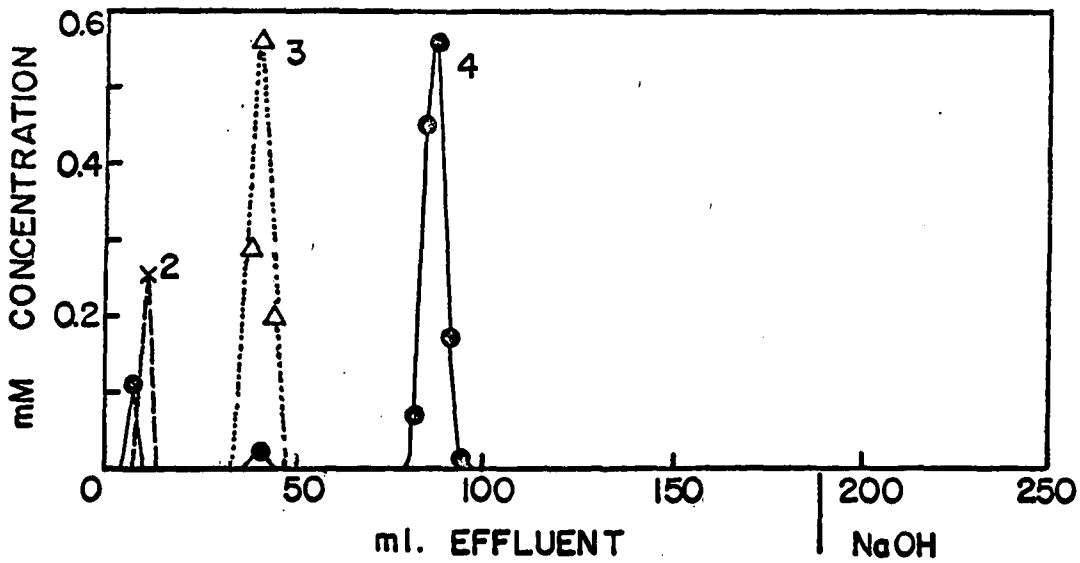
2: Glycocyamidine

3: Ammonia

● Ninhydrin

x Sakaguchi

Δ Jaffé


0.6
0.4
0.2
mM CONCENTRATION
3
4
2
0
50
100
150
200
250
ml. EFFLUENT
NaOH

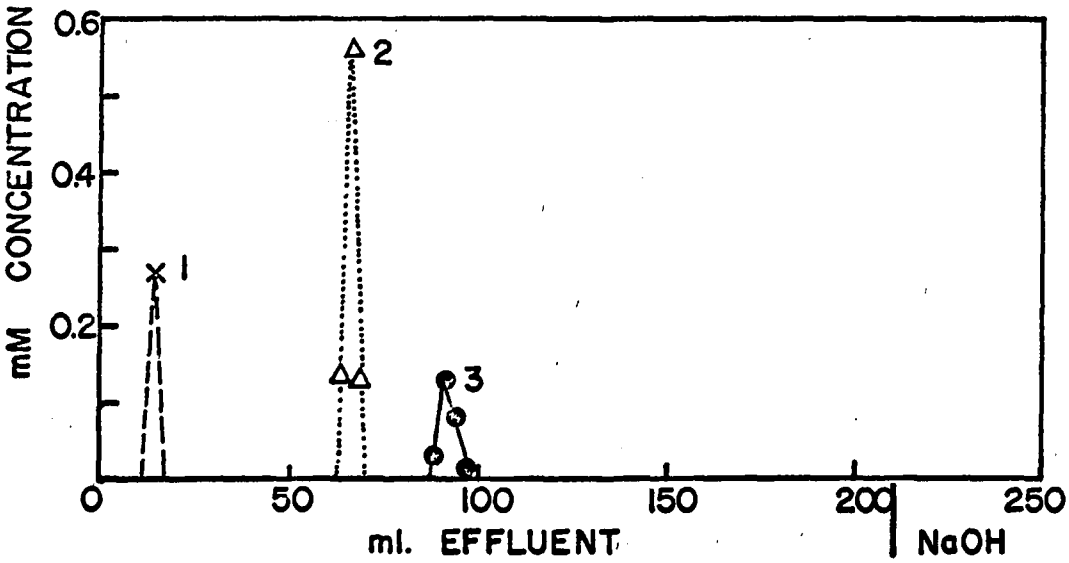

0.6
0.4
0.2
mM CONCENTRATION
2
1
3
0
50
100
150
200
250
ml. EFFLUENT
NaOH

Figure 15. Guanidation of glycyllysine. Fractionation of eight-day reaction mixture

Column: 3.5 x 30 cm. AG 50W-X8

1: Ammonia

2: O-methylisourea

3: Glycylhomoarginine

4: Unknown

5: Glycocyaminylhomoarginine

100 percent reaction of lysine epsilon-amino group

77 percent reaction of glycine amino group

● Ninhydrin

x Sakaguchi

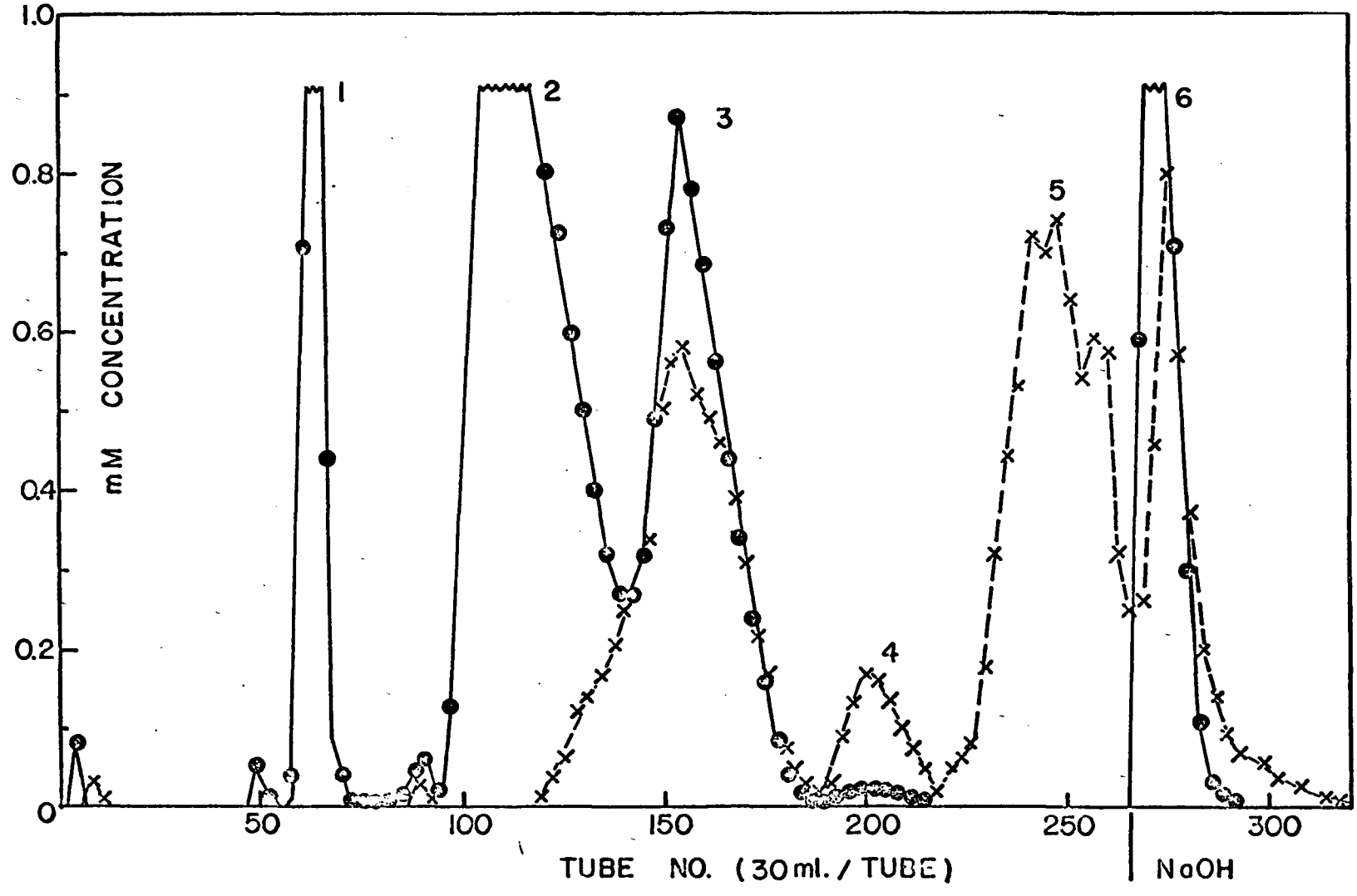

1.0
0.8
0.6
0.4
0.2
0
mM CONCENTRATION
1
2
3
4
5
6
50
100
150
200
250
300
TUBE NO. (30 ml. / TUBE)
NaOH

Figure 16. Guanidation of glycyllysine. Hydrolysates of desalted components from preparative column

Column: 0.9 x 15 cm. Amberlite IR-120

Top: Hydrolysate of peak 3

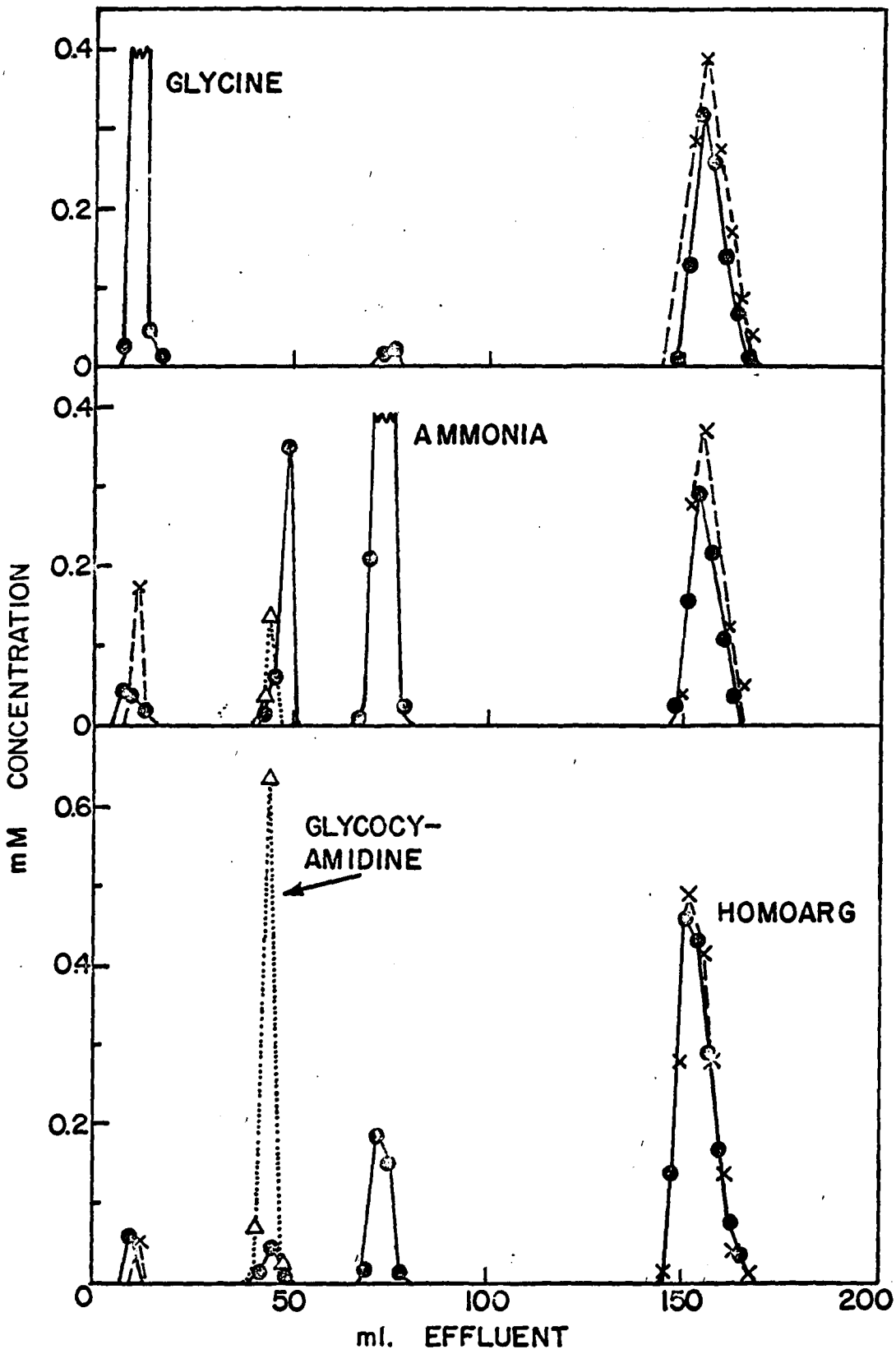
Middle: Hydrolysate of peak 4

Bottom: Hydrolysate of peak 5

● Ninhydrin

x Sakaguchi

Δ Jaffé


GLYCINE
AMMONIA
GLYCOCY-
AMIDINE
HOMOARG
mM CONCENTRATION
ml. EFFLUENT
0.4
0.2
0
0.6
50
100
150
200

G. Lysylglycine

When barium hydroxide was added to the mixture of lysylglycine sulfate and O-methylisourea sulfate, an oil was formed which interfered with centrifugation of the barium sulfate, which remained in suspension. However, the oil disappeared after a few days and centrifugation was then successful in removing precipitated barium sulfate.

Because of this difficulty barium hydroxide was not used in the preparative run.

Reactants:

O-methylisourea sulfate	2.152 gm. (12.5 millimoles)
L-lysylglycine sulfate	0.940 gm. (3.12 millimoles)

The solution containing the reactants was brought to pH 10-11 with 6 N sodium hydroxide and diluted to 25 ml., giving a solution 0.5 M in O-methylisourea and 0.125 M in lysylglycine.

Preparative fractionation of the seven-day reaction mixture revealed the presence of only one major Sakaguchi-positive peak.

Analysis of the desalted and hydrolyzed component in peak 3 from the preparative column revealed the presence of homoarginine and glycine, indicating that the substance in this peak was homoarginylglycine (Figures 17 and 18).

Figure 17. Guanidation of lysylglycine. Fractionation of seven-day reaction mixture
Column: 3.5 x 30 cm. AG 50W-X8

1: Ammonia

2: O-methylisourea

3: Homoarginylglycine

100 percent reaction of lysine epsilon-amino group

No reaction of lysine alpha-amino group

● Ninhydrin

x Sakaguchi

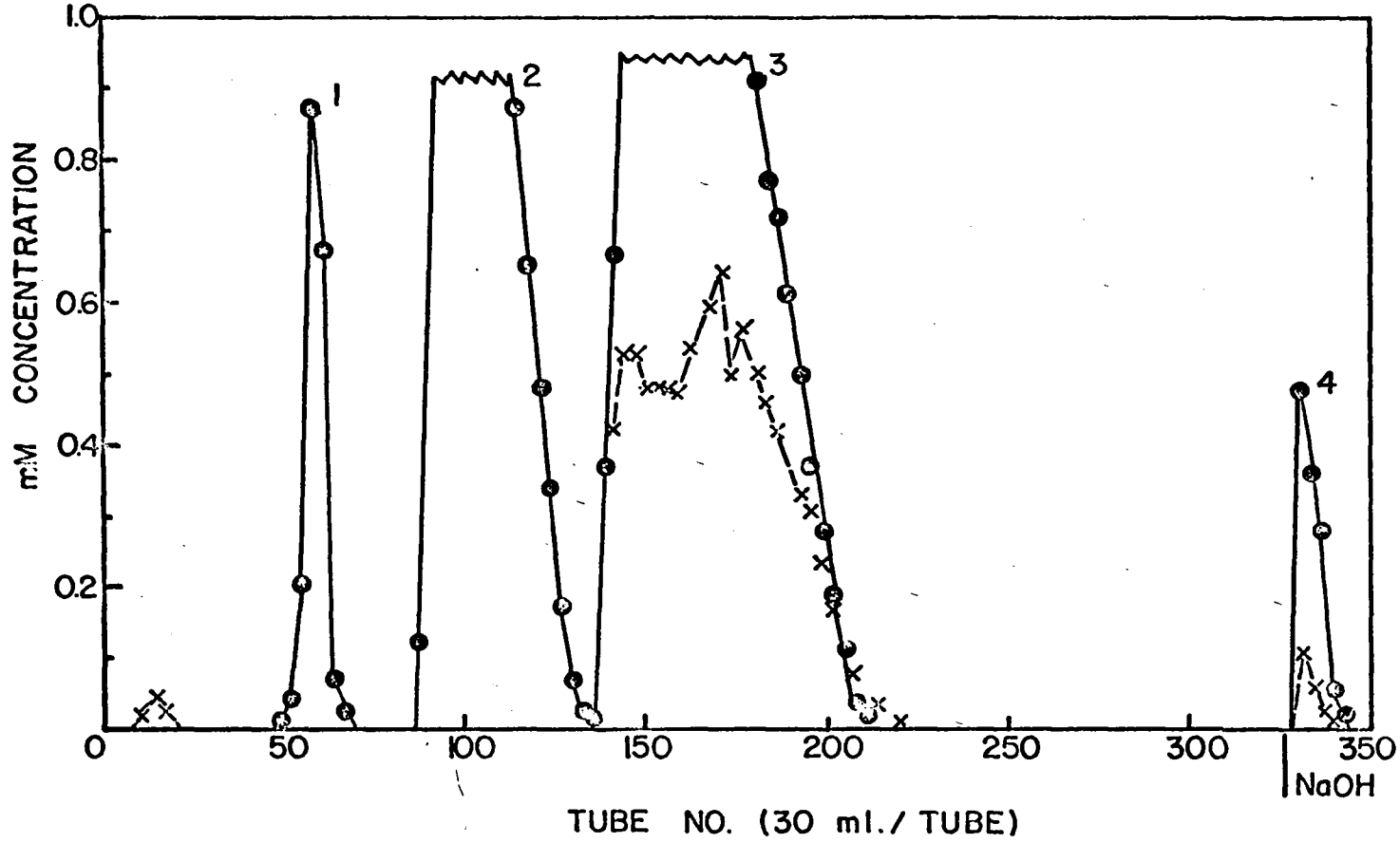

1.0
0.8
0.6
0.4
0.2
0
m.M CONCENTRATION
1
2
3
4
50
100
150
200
250
300
350
NaOH
TUBE NO. (30 ml./ TUBE)

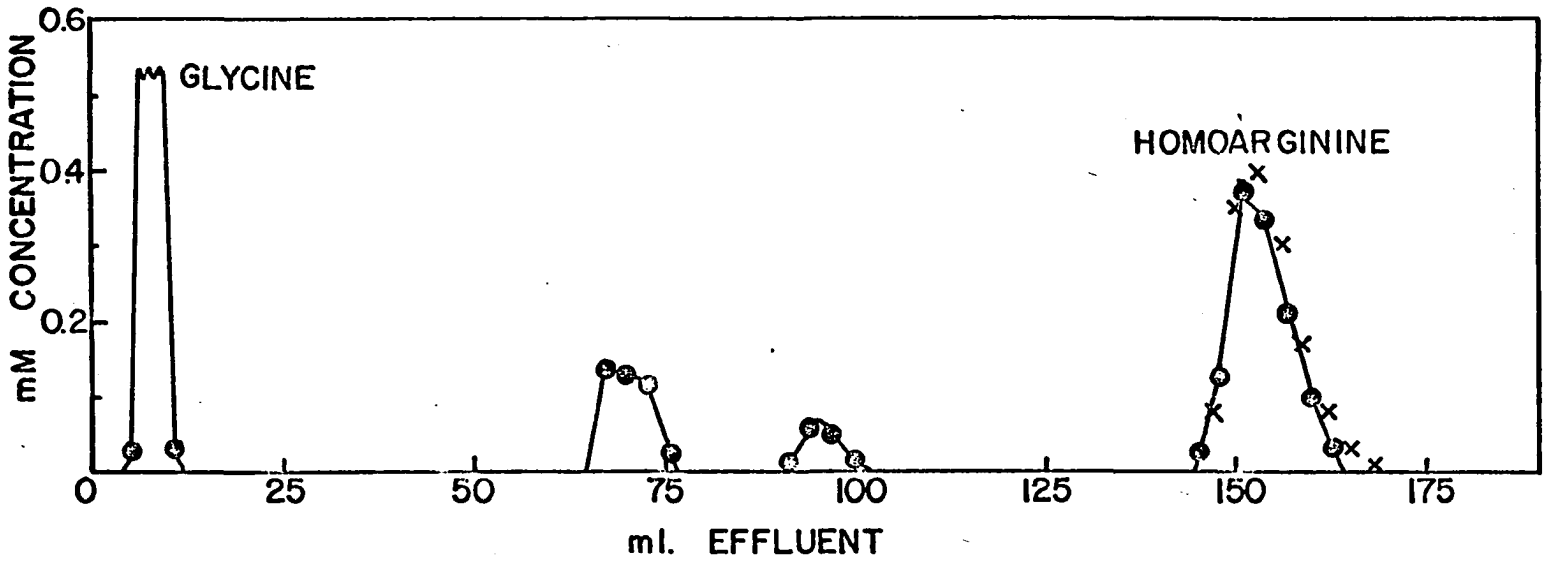
Figure 18. Guanidation of lysylglycine

Hydrolysate of peak 3 of preparative column

Column: 0.9 x 15 cm. Amberlite IR-120

● Ninhydrin

x Sakaguchi


0.6
0.4
0.2
mM CONCENTRATION
GLYCINE
HOMOARGININE
0
25
50
75
100
125
150
175
ml. EFFLUENT

H. Glutathione

In order to determine whether the amino group of an N-terminal glutamic acid residue also reacts with O-methylisourea, this peptide was also subjected to the same guanidation reaction conditions.

Reactants:

O-methylisourea sulfate	0.4304 gm. (2.5 millimoles)
Glutathione (oxidized)	0.3829 gm. (0.625 millimoles)

Sulfate was removed from the guanidating reagent before addition of glutathione. Analysis of the hydrolysate of the six-day reaction mixture with the long column of a Beckman Model 120B amino acid analyzer indicated 33.3 percent conversion of the glutamic acid residue to the guanido derivative. A sample of the hydrolysate gave a strongly positive reaction with the Jaffé reagent, indicating the presence of the cyclized derivative of guanidated glutamic acid.

V. DISCUSSION

The studies with amino acids and peptides reported here show that the alpha-amino group does indeed react with the guanidating reagent O-methylisourea, as determined by column chromatography of reaction mixtures followed by colorimetric analysis of effluent fractions. The alpha-amino groups of lysine (Figure 8), arginine (Figures 9 and 10), glycine (Figures 13, 14, 15, and 16), and glutamic acid were converted into guanido derivatives under the same conditions used in protein guanidation studies. The alpha-amino of arginine reacts relatively rapidly, 51 percent being converted into a guanido group after six days at 3°C. (Figures 9 and 10); that of lysine reacts slowly and only after the epsilon-amino group has reacted. Although no diguanidated lysine was found after eight days at 3°C. (Figure 7), this derivative was found to be the major reaction product after the reaction mixture was stored three weeks in the freezer compartment of a refrigerator (Figure 8). When an attempt was made to guanidate the alpha-amino group of homoarginine itself, no diguanido derivative was found after eight days.

Experiments involving diaminopropionic acid and diaminobutyric acid were performed in order to determine whether the rapid formation of a diguanido derivative was unique to arginine. Although only about 10 percent of these diamino acids was converted to diguanido derivatives after

eight days (Figures 11 and 12), it is possible that more alpha-guanidated products may appear after longer reaction times. Analysis of reaction mixtures during the course of the experiments indicated that the amount of O-methylisourea present after eight days was still more than twice the amount necessary to react with the remaining amino group. It is not clear why an alpha-amino-epsilon-guanido acid is more reactive than either an alpha-epsilon homolog or an alpha-gamma or alpha-beta homolog.

Small amounts of glycocyamine were detected in hydrolysates of guanidated glycyl peptides (Figures 13, 14 and 16). Either cyclization was not complete or some glycocyamidine formed during acid hydrolysis was converted back to the original alpha-guanido acid when the hydrolysate was diluted with pH 2.2 citrate buffer prior to column analysis. Edgar and Shiver have shown that even at this pH some creatinine in a solution is converted to creatine (47).

The investigation reported in this dissertation has dealt mainly with development of analytical techniques applicable to protein guanidation studies. Application of these techniques to guanidated proteins should settle the question of extent of modification, thus clarifying the effects of guanidation on biological activity of enzymes, hormones, and other significant proteins.

Guanidation involves the conversion of one positively charged group into another similarly charged; therefore

proteins whose activity depends on the presence of cationic side groups at specific sites on the macromolecule may retain their biological activity even after guanidation. However, it is also possible that certain proteins may lose activity when amino groups are converted to bulkier guanido groups.

Although some proteins may not be able to form alpha-guanido derivatives due to steric or other factors, there is evidence (mostly indirect) which points to the presence of alpha-guanido acid residues in hydrolysates of certain modified proteins (6, 7, 8, 9, 10, 11).

With slight modifications of the method presented here it would be possible to analyze protein guanidation reactions when more than one N-terminal group is present in the molecule. A 0.9 x 150 cm. column of Amberlite IR-120 (41) could be used to determine extent of reaction of these residues. Although the time involved in operating this column is much greater than is required for the 0.9 x 15 cm. column, separation of acidic and neutral N-terminal residues can be attained with the longer column. Columns on an automatic amino acid analyzer equipped with effluent stream-splitting and fraction collecting devices could also be employed, ninhydrin peaks being observed on the recording chart and Sakaguchi and Jaffé peaks being located by colorimetric tests on the fraction collector samples. The disappearance of

several N-terminal alpha-amino acid residues could then be definitely correlated with appearance of alpha-guanido acid residues or their cyclized derivatives in the protein hydrolysate.

VI. SUMMARY

The reaction between O-methylisourea and amino groups of amino acids and peptides has been investigated in order to determine the relative reactivity of alpha-and epsilon-amino groups. Conditions of reaction and hydrolysis of products were chosen to conform to those used in protein guanidation studies so that results would be directly comparable.

It was shown that the alpha-amino groups of certain amino acids can be easily converted to guanido groups, and that the presence of cyclized derivatives of alpha-guanido acids can be detected in acid hydrolysates by means of the Jaffé colorimetric test.

By means of ion exchange chromatography the various products of amino acid guanidation could be separated, including the diguanido derivatives of ornithine and lysine. The second guanido group is introduced into the molecule much more easily in arginine than in mono-guanidated lysine, 2,3-diaminopropionic acid or 2,4-diaminobutyric acid.

Reaction mixtures and hydrolysates of guanidated peptides were analyzed using the same method. Approximately half of the glycyllysine of an eight-day reaction mixture was converted to the monoguanido derivative, glycylhomoarginine, the rest being converted to the diguanido derivative, glycocyaminylhomoarginine. Lysylglycine was nearly quantitatively converted

to homoarginylglycine after seven days of reaction. Treatment of glutathione with O-methylisourea resulted in the conversion of 33.3 percent of the glutamic acid residues to guanido derivatives after six days of reaction. The insoluble reaction product of glycylglycine precipitated rapidly, allowing the recovery of 20 percent of the theoretical amount of product in three hours by centrifugation.

Preparative-scale columns of Amberlite IR-120 were used for separating large amounts of products, giving elution patterns identical to those obtained with analytical columns. Major components from preparative columns were desalted by employing another column of either Amberlite IR-120 or IRC-50.

VII. LITERATURE CITED

1. Christensen, H. N. J. Biol. Chem. 160, 75 (1945)

2. Geschwind, I. I. and Li, C. H. Biochim. et Biophys. Acta 25, 171 (1957)

3. Chervenka, C. H. and Wilcox, P. E. J. Biol. Chem. 222, 635 (1956)

4. Takahashi, K., Titani, K., Furuno, K., and Ishikura, H. J. Biochem. (Tokyo) 45, 375 (1958)

5. Maekawa, K. and Liener, I. E. Arch. Biochem. Biophys. 91, 108 (1960)

6. Saroff, H. A. and Irreverre, F. Am. Chem. Soc., Abstracts of Papers 126, 91c (1954)

7. Evans, R. L. and Saroff, H. A. J. Biol. Chem. 228, 295 (1957)

8. Habeeb, A. F. S. A. Biochim. et Biophys. Acta 34, 294 (1959)

9. Habeeb, A. F. A. A. Can. J. Biochm. and Physiol. 38, 493 (1960)

10. Vithayathil, P. J. and Richards, F. M. J. Biol. Chem. 235, 1029 (1960)

11. Ellington, E. V. Biochim. et Biophys. Acta 69, 171 (1963)

12. King, H. J. Chem. Soc. 2374 (1930)

13. Kaphammer, J. and Müller, H. Hoppe-Seyler's Z. physiol. Chem. 225, 1 (1934)

14. Schütte, E. Hoppe-Seyler's Z. physiol. Chem. 279, 52 (1943)

15. Mourgue, M. Soc. chim. France, Bull. 181 (1948)

16. Mourgue, M. and Baret, R. Soc. chim. France, Bull. 1224 (1955)

17. Fasold, H., Turba, F. and Wirsching, W. Biochem. Z. 335, 86 (1961)

18. Klee, W. A. and Richards, F. M. J. Biol. Chem. 229, 489 (1957)

19. Roche, J., Mourgue, M. and Baret, R. Soc. chim. biol., Bull. 36, 85 (1954)

20. Minakami, S., Titani, K. and Ishikura, H. J. Biochem. (Tokyo) 45, 341 (1958)

21. Shields, G. S., Hill, R. L. and Smith, E. L. J. Biol. Chem. 234, 1747 (1959)

22. Micheel, F. and Herold, B. Hoppe-Seyler's Z. physiol. Chem. 293, 137 (1953)

23. Hughes, W. J., Jr., Saroff, H. A. and Carney, A. L., Am. Chem. Soc., J. 71, 2476 (1949)

24. Saroff, H. A. and Evans, R. L. Biochim. et Biophys. Acta 36, 511 (1959)

25. Cohn, E. J. Chem. Revs. 28, 413 (1941)

26. Micheel, F. and Heesing, A. Justus Liebigs Ann. Chem. 604, 34 (1957)

27. Takemori, S., Wada, K., Ando, K., Hosokawa, M., Sekuzu, I., and Okunuki, K. J. Biochem. (Tokyo) 52, 28 (1962)

28. Eastoe, J. E. and Kenchington, A. W. Nature 174, 966 (1954)

29. Kenchington, A. W. Biochem. J. 68, 458 (1958)

30. Janus, J. W. in Stainsby, G., ed. Recent advances in gelatin and glue research. p. 214. New York, N. Y., Pergamon Press, Inc. 1958.

31. Betheil, J. J. and Gallop, P. M. Biochim. et Biophys. Acta 45, 598 (196)

32. Morgue, M. and Baret, R. Ann. pharm. franc. 12, 612 (1954)

33. Weil, L. and Telka, M. Arch. Biochem. Biophys. 71, 473 (1957)

34. Kominz, D. R. and Laki, K. Am. Chem. Soc., Abstracts of papers 126, 89c (1954)

35. Tan, P. and Tsao, T. Sci. Sinica (Peking) 6, 1049 (1957)

36. Kay, C. M. and Bailey, K. Biochim. et Biophys. Acta 40, 149 (1960)

37. Ramanchandran, L. K. Biochim. et Biophys. Acta 32, 557 (1959)

38. Joseph, K. T. and Bose, S. T. Central Leather Research Inst., Madras (India), Bull. 5, 399 (1959)

39. Kurtz, A. C. J. Biol. Chem. 180, 1253 (1949)

40. Steib, H. Hoppe-Seyler's Z. physiol. Chem. 155, 292 (1926)

41. Moore, S., Spackman, D. H. and Stein, W. H. Anal. Chem. 30, 1185 (1958)

42. Moore, S. and Stein, W. H. J. Biol. Chem. 211, 907 (1954)

43. Sakaguchi, S. J. Biochem (Tokyo) 37, 231 (1950)

44. Folin, O. and Wu, H. J. Biol. Chem. 38, 81 (1919)

45. Dreze, A., Moore, S. and Bigwood, E. J. Anal. chim. Acta 11, 554 (1954)

46. Winters, J. C. and Kunin, R. Ind. Eng. Chem. 41, 460 (1949)

47. Edgar, G. and Shiver, H. E. Am. Chem. Soc., J. 47, 1179 (1925)

VIII. ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Dr. Walter R. Hearn for suggesting this interesting problem and for providing support, both material and spiritual, throughout the course of this investigation. His encouragement and advice was cheerfully given whenever needed.

To his slave Tom Dixon, who so faithfully and efficiently performed analyses of column effluents, the author shall be eternally grateful.

To his colleagues who provided helpful discussions, the author bequeaths all his glass chromatography columns, fraction collectors, and other pieces of equipment.

Special thanks go to friends and relatives who provided much needed encouragement during times of stress.

This investigation was supported in part by a Public Health Service fellowship (number GMP-19,173) from the Division of General Medical Sciences, Public Health Service.