Hydrogen–Deuterium Exchange of Trypsin and Trypsin Derivatives*

David E. Lenz† and William P. Bryan

ABSTRACT: Hydrogen–deuterium exchange of trypsin and two trypsin derivatives, diisopropylphosphoryl-trypsin and *N*-tosyl-L-lysine chloromethyl ketone-trypsin, was studied by means of infrared spectroscopy. Measurements were made at 23° (0.1 M KCl in D_2O) and at pD values from 2 to 8. Four different classes of exchanging hydrogens were found: a fast class with a pD-dependent rate constant for exchange, a medium class with an essentially pD-independent rate constant for exchange, a slow class with a pD-independent rate constant for exchange, and a "core" class which does not exchange within 24 hr. The fast class could only be observed at pD values around 2 and probably consists of completely exposed peptide hydrogens. The medium and slow classes appear to exchange by a mechanism in which the rate is determined by transconformational exposure of unexposed peptide groups. The rate constants for exchange of the medium or slow classes are essentially the same for trypsin, diisopropylphosphoryl-trypsin, and *N*-tosyl-L-lysine chloromethyl ketone-trypsin.

The numbers of hydrogens per class are essentially the same for the medium, slow or "core" classes for all three substances. These results imply that the medium slow, or "core" peptide hydrogens are essentially structurally identical.

Numerous physical chemical studies have been made on trypsin. Special mention may be made of recent work by d'Albis (d'Albis, 1963, 1964, 1966; d'Albis and Bechet, 1967), by Lazdunski and Delaage (Lazdunski and Delaage, 1965, 1967; Delaage and Lazdunski, 1968), by Luzikov and Troshkina (1966), by Kushner and Bozhkov (1967), and by Arrio (1967). There has also been interest in the possiblity of structural changes of trypsin when substrates or inhibitors are covalently or noncovalently bound at the enzyme's active site. Such interactions have been studied by physical methods (Oppenheimer and Hess, 1963; Benmouyal and Trowbridge, 1966; d'Albis and Bechet, 1967; Johannin and Yon, 1966) and by chemical methods (Spande *et al.*, 1966; Steiner, 1966; Feinstein and Feeney, 1966). The physical technique of hydrogen exchange has been applied to chymotrypsinogen (Wilcox, 1959) and in a very limited way to trypsin (Blout *et al.*, 1961; Uralets and Luzikov, 1966). The work reported here involved a more complete study of hydrogen–deuterium exchange of trypsin by means of infrared spectroscopy. In addition, two covalently inhibited trypsin derivatives. Diisopropylphosphoryl-trypsin and *N*-tosyl-L-lysine chloromethyl ketone-trypsin were also studied in an attempt to learn more about possible structural changes in the trypsin molecule due to covalent inhibition.

Experimental Section

Trypsin was obtained from bovine pancreas as supplied by Worthington Biochemical Corp. It was obtained as twice crystallized, dialyzed salt free, and lyophilized. Two separate lots were used (lot no. TRL-7CB and TRL-6JA). No difference was noted in exchange results obtained from these two lots. Before use the commercial preparations were dialyzed against 10^{-3} M HCl and then lyophilized. Diisopropylphosphoryl-trypsin was a commercial preparation from Worthington (lot no. TDIP-7HA). *N*-Tosyl-L-lysine chloromethyl ketone-trypsin was kindly supplied by Dr. Barbara Sanborn. These derivatives were used as supplied. Deuterium oxide was a commercial preparation obtained from Chemi-Standards with an assay of 99.7% purity.

The activity of trypsin was determined by a modification of the assay developed by Hummel (1959) using *N*-α-benzoyl-L-arginine methyl ester as substrate. The activity of solutions for the reference cell, determined before and after the treatment described below, was found to decrease by 10–15%. The activity of sample solutions was usually found to be unchanged over the time span of the kinetic runs. In addition an active site titration was performed on both lots using the method of Bender *et al.* (1965, 1966) with *p*-nitrophenyl-*N*-benzyloxycarbonyl-L-lysine hydrochloride as substrate. The percentages of trypsin molecules with intact active sites obtained were lot no. TRL-7CB, 54%; lot no. TRL-6JA, 64%.

Two different methods have been used in the in-

* From the Department of Chemistry, Boston University, Boston, Massachusetts 02215. *Received August 16, 1968.* Supported by Grants GB 3499 and GB 6247 from the National Science Foundation.

† Submitted in partial fulfillment for the Ph.D. degree in chemistry, Boston University.

Present address: Department of Chemistry, Georgetown University, Washington, D. C.

vestigation of hydrogen–deuterium exchange in proteins by means of infrared spectroscopy (Hvidt and Nielsen, 1966). They are both based on the fact that the amide II band of a peptide hydrogen in D_2O is shifted from about 1540 cm^{-1} to about 1450 cm^{-1} when the hydrogen is replaced by deuterium. Blout *et al.* (1961) and Hvidt (1963) have used the ratio of absorbances of the peaks of the amide II and amide I bands as a measure of unexchanged hydrogens. Hvidt (1963) and Bensusan and Nielsen (1964) used the absorbance of the amide II band continuously monitored as a function of time. This latter method is suitable when rapidly exchanging hydrogens are to be measured since the spectrum of the exchanging protein need not be scanned.

In our work we have used difference infrared spectroscopy. The absorbance of an exchanging solution of protein was measured against that of a completely exchanged solution under identical conditions. This allows continuous measurement of amide II absorbance automatically corrected for background.

All spectra were measured using a Perkin-Elmer Model 21 double-beam recording infrared spectrometer equipped with a sodium chloride prism and 0.25–5 × continuous-scale expansion. The cells used were a matched pair having calcium fluoride windows and a path length of 0.125 mm. When both cells were filled with D_2O and scanned against each other a good flat base line at 100% transmission was obtained. This indicates that the cells are sufficiently well matched for good difference spectroscopy measurements of the type described here. No leakage of water into the cells during the course of the runs was observed. Special brass water jackets were made for the infrared cells. These jackets were designed so that circulating water could make good thermal contact with the infrared cells and thereby ensure constant temperature during kinetic runs. In this way heating of solutions by the infrared beam was minimized. The temperature of the circulating water was 23.00 ± 0.02°. The temperature of the solutions in the infrared cells could not be measured, but the heating by the infrared radiation was estimated to be below 0.5°.

Preliminary scans of the spectrum of trypsin showed that the peak of the amide II band was at a wavelength of 6.500 μ (1538 cm^{-1}). This wavelength was chosen for all kinetic runs. Additional preliminary experiments established the conditions for complete exchange of trypsin peptide hydrogens. A 1% solution of trypsin in D_2O showed a transmission of 95–96% upon complete exchange when measured against D_2O. Further heating or exposure to D_2O gave no further change in transmission. Conditions for complete exchange were established at the various pD values of the kinetic runs. Before each kinetic run the solution for the reference cell was heated at 55° for 20 min to 2 hr depending upon the pD. This ensured that exchange was complete in the reference solution.

Samples for use in kinetic runs were prepared from a 1% solution of trypsin or its derivatives in water. The solution was adjusted to the approximate pH of a run by addition of HCl or NaOH. Aliquots of 500 μ were then taken and lyophilized. These samples were stored at 4° until used but never for more than 1 week. At the start of a run one of the aliquots was dissolved in 500 μl of D_2O (0.1 M in KCl) and then heated as described above in order to exchange all exchangeable hydrogens. After heating, the pD of this reference solution was determined using ordinary pH electrodes and a meter standardized with an H_2O buffer solution. Meter readings were corrected to pD values according to Glascoe and Long (1960) where

$$\text{pD} = \text{meter reading} + 0.40 \qquad (1)$$

The concentration of the reference solution was obtained by dilution of a sample with water and measurement of absorbance at 280 mμ using 1-cm cells. Concentration of trypsin in milligrams per milliliter was calculated by multiplication of absorbance by 0.65.

A portion of the reference solution was placed in the reference cell. To start a run 500 μl of D_2O (0.1 M in KCl) was added to a second lyophilized sample. Zero time was taken upon solution of this sample. A portion of the solution was quickly added to the sample cell and transmission at 1538 cm^{-1} was followed as a function of time. Kinetics were followed for about 2 hr. A final reading was taken at 24 hr. A scale expansion of two to four times was generally used.

Data were obtained in terms of chart reading (linear transmittance scale) *vs.* time. The data were converted into absorbance *vs.* time taking scale expansion into account. Generally duplicate or triplicate runs were made at all pD values. Absorbance values from different runs never disagreed by more than about 20%. Data from all runs at a given pD were plotted together and treated as described below.

In order to obtain values of specific rate constants for exchange of the various classes of exchanging groups (k_m values), certain arbitrary assumptions must be made for solutions of trypsin and its derivatives in D_2O. We assume that all exchangeable hydrogens (taken as all peptide hydrogens and *trans* primary amide hydrogens) absorb equally well at 1538 cm^{-1} and that this absorbance is independent of pD. If exchange behavior is described by the equation

$$h_i = \sum_m n_m e^{-k_m t} \qquad (2)$$

where h_i represents the number of exchangeable hydrogens per molecule not exchanged at time t and n_m represents the number of hydrogens per molecule in class m characterized by exchange constant k_m, then the above assumptions lead to the relationship

$$A_i = \sum_m A_m e^{-k_m t} \qquad (3)$$

where A represents absorbance. Use of eq 2, which is derived on the assumption that all hydrogens in a protein molecule exchange independently of each other, implies neglect of the possibility that rates of exchange may be dependent upon degree of deuteration during the course of exchange (Hvidt and Nielsen, 1966).

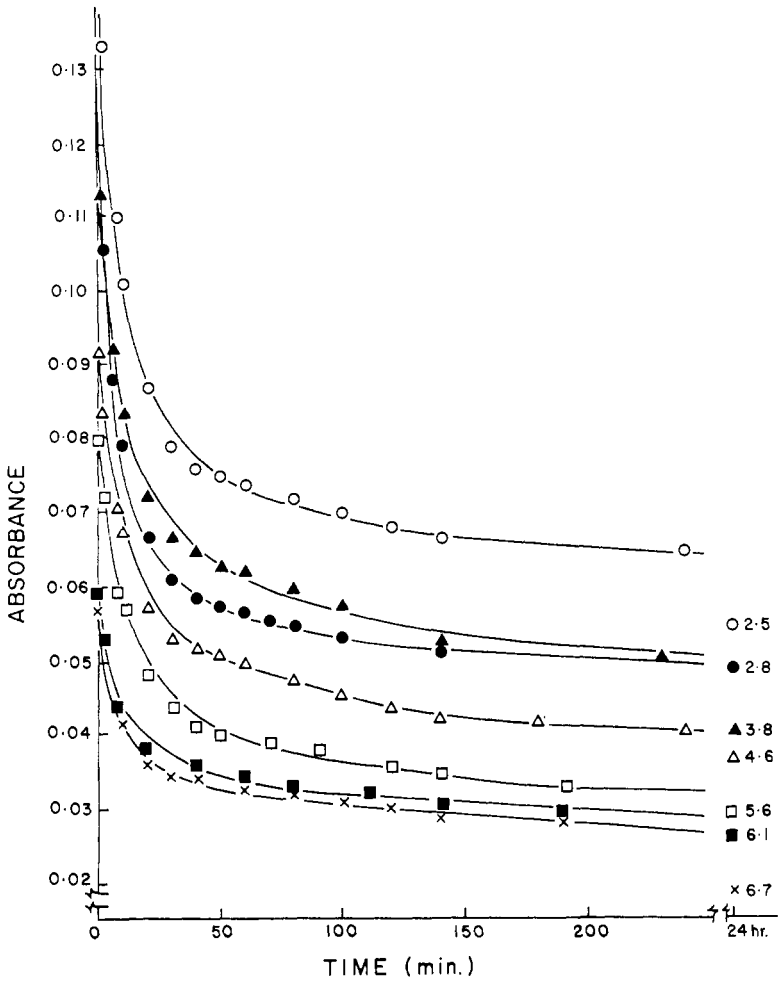

FIGURE 1: A_i *vs.* time for the amide II band (1538 cm^{-1}) of 1% trypsin in D_2O (0.1 M in KCl) at various values of pD as indicated. Temperature was 23.0 ± 0.5°.

Addition assumptions allow calculation of h_i and n_m from experimental absorbance data. If ϵ, the mean molar extinction coefficient per exchangeable hydrogen, is known, measured values of A_i and A_m can be converted into h_i and n_m. In calculating ϵ we assume that all exchanging peptide and *trans* amide hydrogens in trypsin can be observed at pD 2.5. The greatest absorbance changes are observed at this pD. Model compound studies (Hvidt and Nielsen, 1966) indicate that rates of exchange of completely exposed hydrogens should be observable at a pD of 2.5. Only peptide and *trans* primary amide hydrogens are assumed to contribute to the amide II band (Miyazawa, 1967). Previous hydrogen exchange studies have not considered primary amide hydrogens as contributing to the amide II band. Trypsin contains 222 peptide hydrogens and 27 primary amide groups (Walsh and Neurath, 1964; Mikes *et al.*, 1967). Therefore, 249 exchangeable hydrogens are assumed to contribute to the amide II band at the start of an exchange experiment. Values of ϵ were calculated from eq 4

$$\epsilon = \frac{\sum_m A_m}{\frac{c}{23{,}800} \times 1.25 \times 10^{-2} \times 249} \qquad (4)$$

where the Σ term represents the absorbance at the start of an exchange experiment and c is the concentration of trypsin in milligram per milliliter. Values of the Σ term were obtained by two methods: graphical extrapolation and computer calculation. Values of ϵ obtained were: 94 M^{-1} cm^{-1} (graphical extrapolation) and 101 M^{-1} cm^{-1} (computer calculation). These values were also arbitrarily used in treating the exchange data for the trypsin derivatives.

Experimental data were resolved graphically (eq 3) using points from smooth curves drawn through semilogarithmic plots of A_i *vs.* t. The data were also analyzed by means of a computer program developed by Segal and Harrington (1967). Equally spaced points from smooth curves drawn through plots of A_i *vs.* t were taken and treated as described in their paper. Before both types of analysis, 24-hr values of A_i, corresponding to A_m values for "core" hydrogens, were subtracted from measured A_i values. After this subtraction both methods gave only three classes of hydrogens: a fast class seen only a low pD values, a medium class, and a slow class. A_m values were converted into n_m values using ϵ determined as described above. These n_m values cannot be of high absolute accuracy since many assumptions have been made in the determination of ϵ. The k_m values are somewhat more accurate since they are obtainable from absorbance *vs.* time data by use of fewer assumptions.

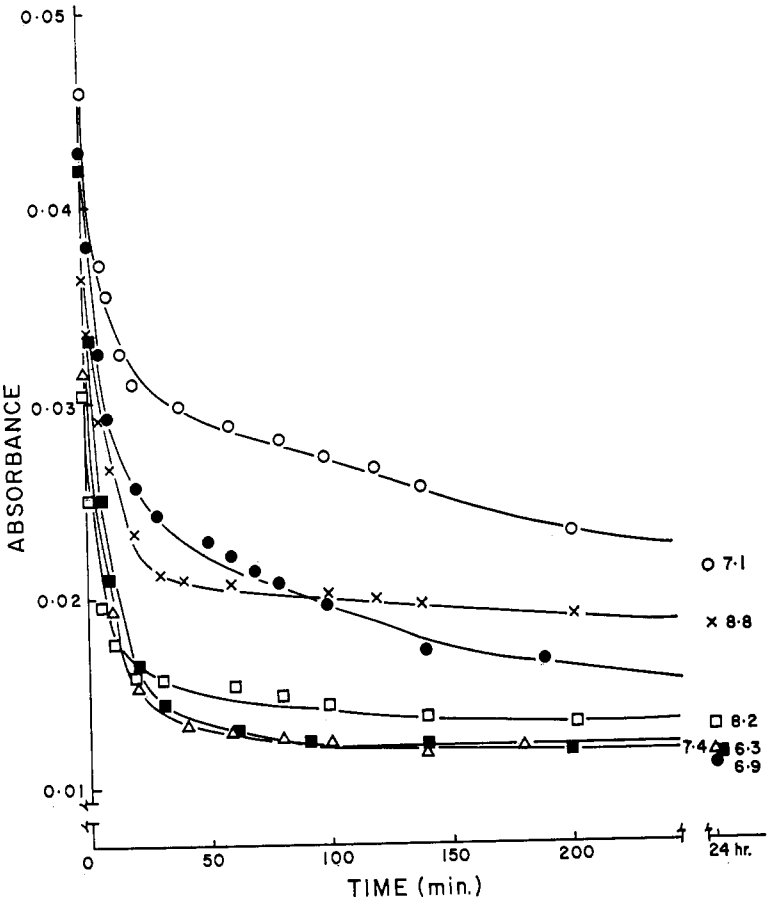

FIGURE 2: A_i *vs.* time for the amide II band (1538 cm^{-1}) of 1% trypsin in D_2O (0.1 M in KCl) at various values of pH as indicated. Temperature was 23.0 ± 0.5°.

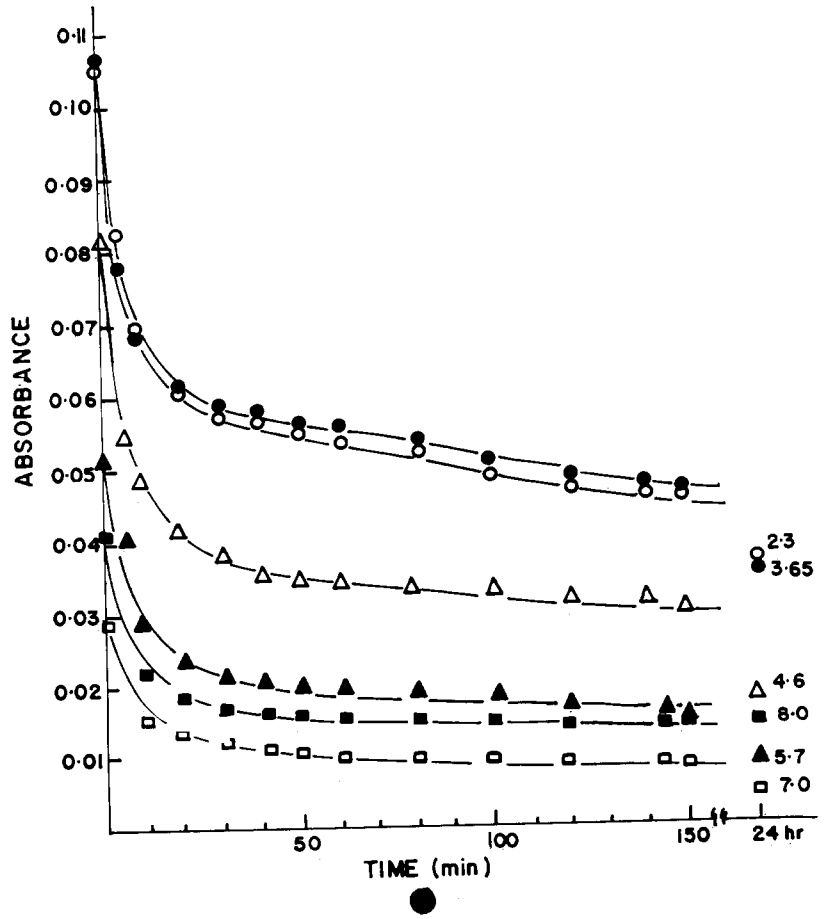

FIGURE 3: A_i *vs.* time for the amide II band (1538 cm^{-1}) of 1% diisopropyl-phosphoryl-trypsin in D_2O (0.1 M in KCl) at various values of pD as indicated. Temperature was 23.0 ± 0.5°.

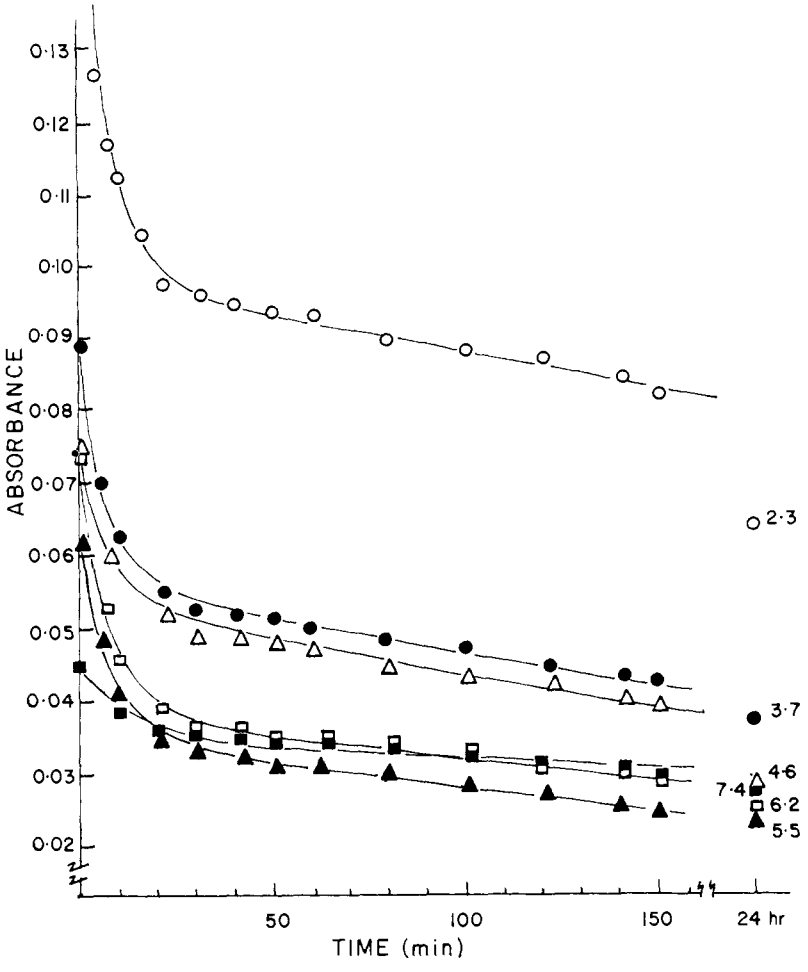

FIGURE 4: A_i *vs.* time for the amide II band (1538 cm^{-1}) of 1% *N*-tosyl-L-lysine chloromethyl ketone-trypsin in D_2O (0.1 M in KCl) at various values of pD as indicated. Temperature was 23.0 ± 0.5°.

Results

Plots of A_i *vs.* t for 1% trypsin solutions in D_2O (0.1 M in KCl) are given in Figures 1 and 2. Similar data for diisopropylphosphoryl-trypsin and *N*-tosyl-L-lysine chloromethyl ketone-trypsin are given in Figures 3 and 4. Below pD values of about 6 an increase in pD is paralleled by a decrease in the total absorbance observed and by a decrease in n_m values for core hydrogens. Above pD 6 the picture is confusing and the h_m values for core hydrogens appear to vary somewhat erratically with pD. In this region experimental error is a greater percentage of observed absorbance and could obscure real effects. Nevertheless it appears that even at pD values in the region 7–9, there are some hydrogens which have not exchanged, even after 24 hr at 23°.

The results of the computer calculations for trypsin, diisopropylphosphoryl-trypsin, and *N*-tosyl-L-lysine chloromethyl ketone-trypsin are given in Tables I–III. Results of graphical analysis are in general agreement with computer calculations. Since graphical results are obtained in a less objective way, they will not be given in detail. An over-all comparison of results obtained from both methods of analysis is given in Table IV.

The data in Table I indicate that trypsin shows three classes of exchanging hydrogens plus a "core" class which is not exchanged after 24 hr at 23°. The fast class is only seen at pD values of 2.5 and 2.8. At higher pD values k_{fast} becomes too large for measurement by the present technique. Values of k_{medium} are obtainable at all experimental conditions. Computer calculated data indicate that k_{medium} increases somewhat as pD increases.[1] The slow class has a constant value of k_{slow} over the entire pD range of the measurements.

Data in Tables II and III show two classes of exchanging hydrogens plus a "core" class. Values of k_{medium} are somewhat higher at pD's of 2.3 and 3.7 as though fast and medium class might both be present but unresolved. There appears to be somewhat of an increase in k_{medium} values in the pD range 4.6–8.0[1] The rate constant for exchange of the slow class is constant over the entire pD range. Reference to Table IV shows that both methods of analysis of the data indicate agreement of mean k_m values for trypsin and both derivatives.

Figures 5–7 show plots of n_m against pD for medium,

[1] This increase in k_{medium} is not seen in graphically calculated results. Graphical calculation indicates essentially constant k_{medium} over the entire pD range. We feel that graphical calculation may be more correct here since smooth curves drawn through log A_i *vs.* t plots would be expected to be more reliable than smooth curves drawn through A_i *vs.* t plots at low values of t where A_i is rapidly changing with time.

TABLE I: Rate Constants (k_m, min^{-1}) and Number of Hydrogens in Each Class (n_m, Hydrogens per Molecule) for the Exchange of Trypsin at 23° in 0.1 M KCl at Various pD Values. Computer Calculations.

pD	k_{fast}	n_{fast}	k_{medium}	n_{medium}	k_{slow}	n_{slow}	n_{core}	Σn_m[a]
2.5	2.0×10^{-1}	15	2.5×10^{-2}	109	7.2×10^{-3}	30	95	249
2.8	3.0×10^{-1}	43	3.9×10^{-2}	69	8.9×10^{-3}	25	85	222
3.8	*b*	*b*	4.1×10^{-2}	71	7.8×10^{-3}	48	72	191
4.6	*b*	*b*	4.6×10^{-2}	63	8.1×10^{-3}	32	65	160
5.6	*b*	*b*	2.9×10^{-2}	59	7.6×10^{-3}	20	52	131
6.1	*b*	*b*	7.2×10^{-2}	31	8.1×10^{-3}	21	47	99
6.3	*b*	*b*	5.3×10^{-2}	37	9.0×10^{-3}	5	21	63
6.7	*b*	*b*	9.2×10^{-2}	42	7.3×10^{-3}	29	33	104
6.9	*b*	*b*	1.0×10^{-1}	27	7.8×10^{-3}	27	21	75
7.1	*b*	*b*	1.0×10^{-1}	37	7.6×10^{-3}	19	37	93
7.4	*b*	*b*	3.8×10^{-2}	24	9.0×10^{-3}	5	21	50
8.2	*b*	*b*	1.1	5	9.1×10^{-3}	8	22	35
8.8[c]	*b*	*b*	5.0×10^{-2}	28	7.3×10^{-3}	6	31	65

[a] Σn_m for trypsin at pD 2.5 assumed equal to 249 hydrogens/molecule. All other n_m values calculated using ϵ derived on this basis. [b] Exchange too fast to measure. [c] In the presence of 0.2 M "Tris" buffer to minimize pD changes due to autolysis.

TABLE II: Rate Constants (k_m, min^{-1}) and Number of Hydrogens in Each Class (n_m, Hydrogens per Molecule) for the Exchange of Diisopropylphosphoryl-trypsin at 23° in 0.1 M KCl at Various pD Values. Computer Calculations.

pD	k_{medium}	n_{medium}	k_{slow}	n_{slow}	n_{core}	Σn_m[a]
2.3	9.0×10^{-2}	82	7.6×10^{-3}	44	63	189
3.7	1.5×10^{-1}	83	7.8×10^{-3}	48	62	193
4.6	3.4×10^{-2}	47	7.1×10^{-3}	34	31	112
5.7	5.9×10^{-2}	39	7.4×10^{-3}	24	16	79
7.0	6.8×10^{-2}	20	7.4×10^{-3}	14	7	41
8.0	5.9×10^{-2}	29	9.8×10^{-3}	6	25	60

[a] Σn_m for trypsin at pD 2.5 assumed equal to 249 hydrogens/molecule. All other n_m values calculated using ϵ derived on this basis.

slow, and "core" classes of trypsin and its derivatives. The data scatter rather badly and it is difficult to estimate errors in n_m. No significant differences in n_m values in any class for trypsin, diisopropylphosphoryl-trypsin, or *N*-tosyl-L-lysine chloromethyl ketone-trypsin are observed.[2] Small differences in n_m values would not be detected by this rather imprecise infrared method.

Discussion

Hvidt (1964) and Hvidt and Nielsen (1966) have discussed a mechanism first proposed by Linderstrom-Lang (1955) for hydrogen exchange in proteins. The results for trypsin can be discussed in terms of the Linderstrom-Lang mechanism. This mechanism postulates exchange as occurring through some intermediate structure I which arises by means of a transconformational change of the native structure N.

$$\begin{gathered} D_2O + N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I(D_2O) \\ I(D_2O) \xrightarrow{k_3} \text{exchange} \end{gathered} \tag{5}$$

Exchangeable hydrogens in I are assumed to be completely exposed to solvent. We further assume that the usual steady state approximation is valid for $I(D_2O)$. Then the value of k_m, the measured rate constant for exchange, is given by

$$k_m = \frac{k_1 k_3}{k_2 + k_3} \tag{6}$$

A protein may be considered as made up of several regions, each of which is characterized by distinct values of k_m and n_m.

Two limiting cases of eq 6 are of interest. If $k_3 >> k_2$,

[2] If the experimental results are treated using different values of ϵ for trypsin, diisopropylphosphoryl-trypsin and *N*-tosyl-L-lysine chloromethyl ketone-trypsin the agreement in n_m values for these three substances is improved.

TABLE III: Rate Constants (k_m, min^{-1}) and Number of Hydrogens in Each Class (n_m, Hydrogens per Molecule) for the Exchange of *N*-Tosyl-L-Lysine Chloromethyl ketone-trypsin at 23° in 0.1 M KCl at Various pD Values. Computer Calculations.

pD	k_{medium}	n_{medium}	k_{slow}	n_{slow}	n_{core}	Σ_m[a]
2.3	9.0×10^{-2}	103	7.1×10^{-3}	76	99	278
3.7	4.4×10^{-2}	53	7.5×10^{-3}	29	65	147
4.6	2.3×10^{-2}	40	7.1×10^{-3}	34	50	124
5.5	1.1×10^{-1}	78	8.3×10^{-3}	22	40	140
6.2	9.1×10^{-2}	52	8.0×10^{-3}	24	44	120
7.4	3.6×10^{-1}	45	7.6×10^{-3}	16	48	109

[a] Σn_m for trypsin at pD 2.5 assumed equal to 249 hydrogens/molecule. All other n_m values calculated using ϵ derived on this basis.

TABLE IV: Mean Values of Rate Constants for Exchange of Trypsin and Derivatives at 23° in 0.1 M KCl (pD 2–9).

Substance	Class of Exchangeable Hydrogens	Mean k_m (min^{-1}) Computer	Graphical
Trypsin	Medium	5.7×10^{-2}[a]	12.2×10^{-2}
	Slow	8.0×10^{-3}	6.8×10^{-3}
DIP-trypsin	Medium	5.9×10^{-2}	12.6×10^{-2}
	Slow	7.9×10^{-3}	2.9×10^{-3}
TLCK-trypsin	Medium	6.6×10^{-2}	14.0×10^{-2}
	Slow	7.6×10^{-3}	5.7×10^{-3}

[a] Results at pD 8.2 excluded.

then $k_m = k_1$. The rate of exchange is given by the rate of a transconformational opening of the protein region. This limiting case can be designated as EU_1 (W. P. Bryan, unpublished data). If $k_2 >> k_3$, $k_m = (k_1/k_2)k_3$. In this case I(D_2O) is formed and removed many times before exchange can occur. This limiting case can be designated as EU_2.

Special interest resides in the estimation of k_3. Studies of model compounds (Hvidt and Nielsen, 1966) have indicated that k_3 is approximately given by

$$k_3 = k_O + k_H(H^+) + k_{OH}(OH^-) \quad (7)$$

The exchange of exposed peptide hydrogens is acid and base catalyzed. Reference to eq 7 shows that in the EU_2 case k_m should be pH dependent even when k_1/k_2 is pH independent. In the EU_1 case k_m is pH independent provided k_1 is.

The best model substance corresponding to an I region in a protein is difficult to choose. Hvidt and Nielsen have suggested randon coil poly-DL-alanine in water as such a substance. We can use data for the exchange of poly-DL-alanine in D_2O (Bryan and Nielsen, 1960) in discussing our trypsin results.

Table I shows that trypsin has a fast class of exchanging hydrogens which is only seen at pD values of 2.5 and 2.8. The data in Tables II and III also indicate the possibility of unresolved fast hydrogens at the lowest pD values. The poly-DL-alanine data indicate that k_3 for poly-DL-alanine has a minimum value of 1.1×10^{-1} min^{-1} at pD 3.2 and 22°. Since trypsin is positively charged at these low pD values, we would expect that any completely exposed hydrogens would exchange with about the same value of k_m as for poly-DL-alanine (Leichtling and Klotz, 1966) and that the pD value for minimum k_m (here $\cong k_3$) would be shifted somewhat below pD 3.2. This is because the positive charge on trypsin would tend to enhance OD^- catalysis and diminish D^+ catalysis in comparison with poly-DL-alanine. The k_{fast} values obtained for trypsin ($2–3 \times 10^{-1}$ min^{-1}) are of the right order of magnitude and the minimum in rate for the fast hydrogens appears to occur at a pD value less than 3.0. Thus these fast hydrogens apparently correspond to exposed hydrogens of the trypsin molecule.

Inspection of Tables I–IV indicates that k_{medium} is essentially constant for trypsin and the two derivatives. Furthermore k_{medium} values for all three substances are essentially the same. The same statements apply to k_{slow}. Constancy of these k_m values is consistent with an EU_1 type of mechanism ($k_m = k_1$) for these two classes. Evidently k_1 for both medium and slow classes is essentially pD independent. The possibility that $k_m = (k_1/k_2)k_3$; and that increases in k_3 due to pD increases (eq 7) are compensated for by decreases in k_1/k_2, with the result that k_m is approximately constant, is unlikely. This is because Lazdunski and Delaage (1967) have shown that for trypsin the equilibrium constant for thermal denaturation is independent of pH at pH values from 3 to 8. Since k_1/k_2 is similar to such an equilibrium constant it should also be essentially pH independent. Therefore if exchange were by an EU_2 mechanism k_m would not be expected to be independent of pD.

Figures 5–7 show decreases in n_m values as pD is increased. This is not in agreement with the EU_1 type of Linderstrom-Lang mechanism which predicts constant n_m values. It is unlikely that exchange in trypsin is by an EU_2 type of Linderstrom-Lang mechanism and that the decrease in Σn_m is due to various classes becoming too

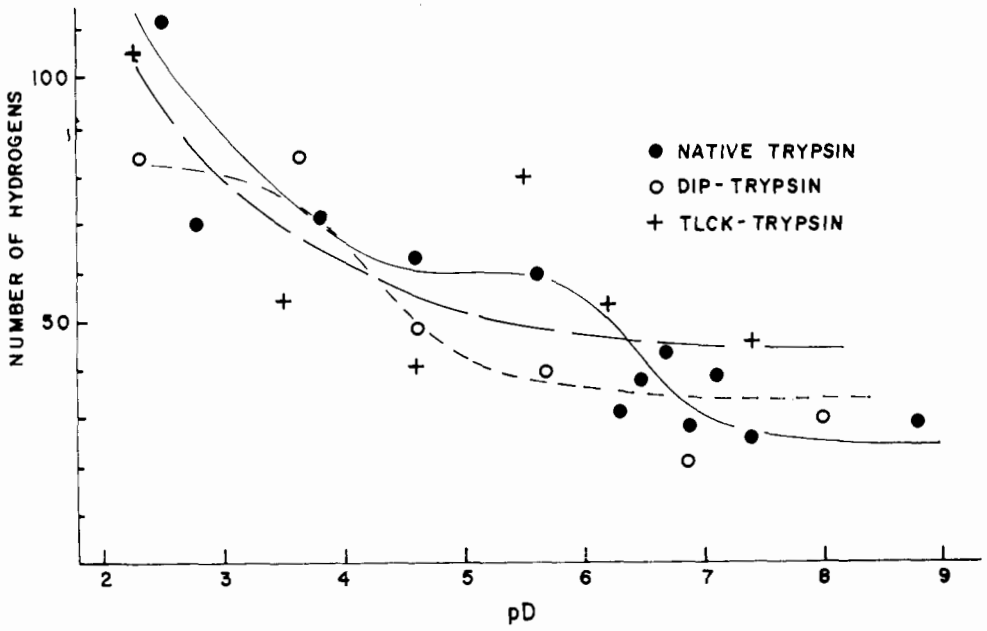

FIGURE 5: Number of hydrogens per class, n_m, for medium class of exchanging hydrogens as a function of pD for trypsin, diisopropylphosphoryl-trypsin, and *N*-tosyl-L-lysine chloromethyl ketone-trypsin.

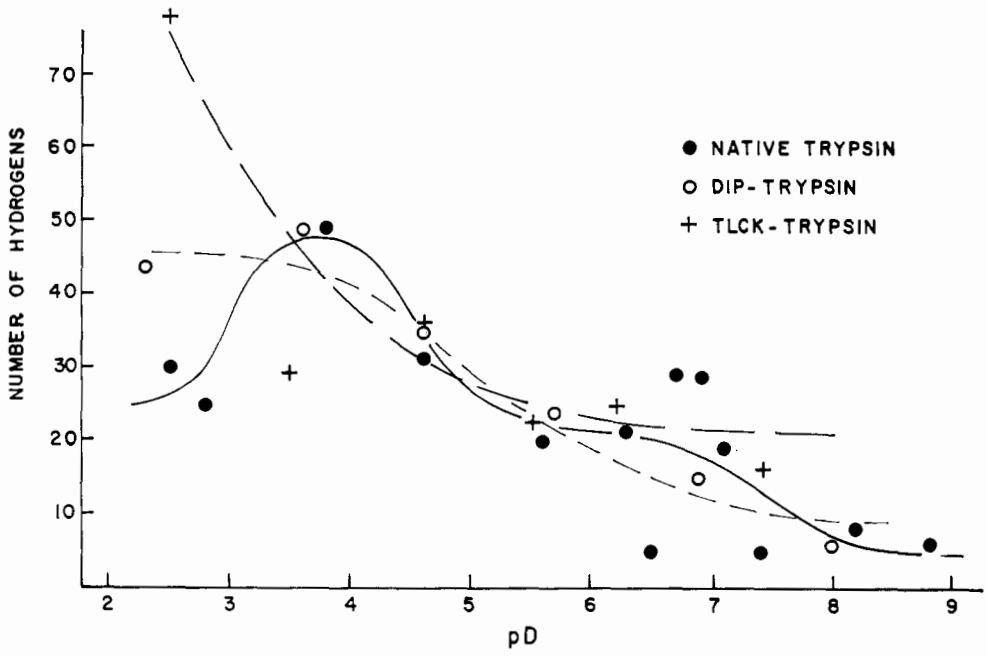

FIGURE 6: Number of hydrogens per class, n_m, for slow class of exchanging hydrogens as a function of pD for trypsin,diisopropylphosphoryl-trypsin, and *N*-tosyl-L-lysine chloromethyl ketone-trypsin.

fast to measure as pD increases. In general the n_m values observed cannot be fitted by such a model[3] and the k_m values are essentially constant.

[3] The data for diisopropylphosphoryl-trypsin (Table II), but not the data for trypsin or *N*-tosyl-L-lysine chloromethyl ketone-trypsin, are in approximate agreement with an EU_2 mechanism. However, we favor an EU_1 mechanism for all three substances because the k_m values for diisopropylphosphoryl-trypsin are pD independent, and the k_m values for all three substances are in good agreement with each other.

The changes in n_m values can be explained if structural changes of the trypsin molecule are taken into account. Work of Lazdunski and Delaage (1967) has indicated the presence of four structurally distinct forms of trypsin in the acid pH region. The transitions and their midpoints are IV → III (pH 1.5), III → II (pH 3.0), II → I pH ~ 5.0). Similar transitions would be expected in D_2O, probably at roughly the same or slightly higher pD values. Our data can be explained, then, by postulating that in the transitions III → II and II → I there are partial unfoldings of the trypsin structure resulting in decreased

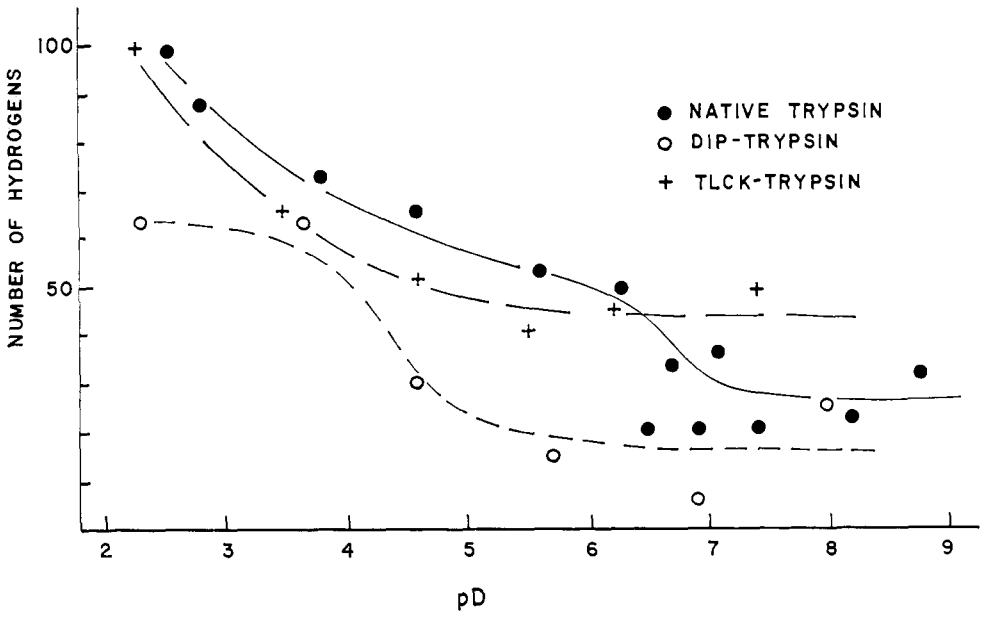

FIGURE 7: Number of hydrogens per class, n_m, for "core" class of exchanging hydrogens as a function of pD for trypsin, diisopropylphosphoryl-trypsin, and *N*-tosyl-L-lysine chloromethyl ketone-trypsin.

n_m and unchanged k_m values. The trypsin derivatives also appear to show such behavior.

Other physical chemical studies give some indication of a "tightening" of the trypsin structure as pH increases. Studies using autolysis (Lazdunski and Delaage, 1965), optical rotation (Lazdunski and Delaage, 1965; d'Albis, 1966), and ultraviolet spectroscopy (d'Albis, 1964; Lazdunski and Delaage, 1965, 1967) as criteria for trypsin "compactness" indicate increased stability as pH is increased from 2 to 8. However, it is difficult to see just how these methods relate to "compactness" of trypsin, whereas our interpretation regarding n_m values is straightforward in this regard. Hydrogen exchange is a better method for such study since n_m values are directly determined. We might also mention that the sedimentation coefficient for trypsin is the same at pH 2.4 and 8 (Nord and Bier, 1953).

If our interpretation is correct, our results indicate that at least three forms of trypsin can be present under our experimental conditions.[4] One form (T) would correspond to all exchanging regions in N forms. The other two would correspond to the medium (T′) and slow (T″) regions in I forms. This can be indicated as

$$T'' \underset{k_2''}{\overset{k_{slow}}{\rightleftharpoons}} T \underset{k_2'}{\overset{k_{medium}}{\rightleftharpoons}} T' \qquad (8)$$

The extent of formation of T′ and T″ is unknown since k_2' and k_2'' have not been determined

Luzikov and Troshkina (1966) have measured the kinetics of reversible and irreversible inactivation of trypsin at pH 3 and higher temperatures. They formulate the scheme

$$D_{ir} \xleftarrow{\beta_3} N \underset{\beta_2}{\overset{\beta_1}{\rightleftharpoons}} D_r \qquad (9)$$

Their measurements of β_1 and β_3 when extrapolated to 23° (by means of their experimentally determined activation energies) give: $\beta_1 = 6 \times 10^{-7}$ min^{-1} and $\beta_3 = 1 \times 10^{-5}$ min^{-1}. These rate constants are much slower than k_{slow} and k_{medium}. Evidently the thermal unfolding of trypsin may involve more than one step.

The positions for the attachment of inhibitors to form inhibited trypsin derivatives are well known. Diisopropylphosphoryl-trypsin has a diisopropylphosphoryl group on serine 183 and *N*-tosyl-L-lysine chloromethyl ketone-trypsin has histidine 46 alkylated by *N*-tosyl-L-lysine chloromethyl ketone. Both of these residues are involved in enzymatic activity. Studies in the literature indicate some structural differences between trypsin and its derivatives. Oppenheimer and Hess (1963) have shown differences between diisopropylphosphoryl-trypsin and trypsin by means of ultraviolet difference spectroscopy. Delaage and Lazdunski (1968) have measured denaturation rates in 6 M urea. Rates of denaturation increase in the order: *N*-tosyl-L-lysine chloromethyl ketone-trypsin < trypsin < diisopropylphosphoryl-trypsin. Ultraviolet difference spectra are also shown by nonconvalent complexes at the trypsin active site (Benmouyal and Trowbridge, 1966; d'Albis and Bechet, 1967; Johannin and Yon, 1966). These results generally indicate involvement of tyrosine (Oppenheimer and Hess, 1963; d'Albis and Bechet, (1967) but noninvolvement of tryptophan (Spande *et al.*, 1966).

[4] Provided all trypsin molecules present are essentially structurally equivalent. There is some evidence that this may not be so. For example, all of our trypsin is not enzymatically active and two forms of trypsin can be separated by salt fractionation (d'Albis, 1963).

The results reported here indicate that, within the rather low precision of our measurements, no significant differences are obtained in k_m values between trypsin and the derivatives for medium or slowly exchanging hydrogens and no significant differences are obtained in n_m values for medium, slow, or "core" hydrogens. Thus large structural changes between trypsin and its two derivatives are ruled out. The differences observed by other physical techniques are probably due to local effects. Hydrogen–deuterium exchange studies can detect structural changes due to interactions at enzyme active sites (Hvidt and Kagi, 1963; DiSabto and Ottesen, 1965). That such changes have not been detected in the present study indicates that they cannot be very extensive.

This conclusion is in agreement with the recent X-ray results for chymotrypsin (Mathews *et al.*, 1967). These results indicate only minor differences in structure between tosyl-α-chymotrypsin (tosyl group at serine 195) and α-chymotrypsin itself.

Acknowledgment

The computer program used in calculating our results was that used by Segal and Harrington (1967). It was generously furnished by Mr. Peter Hauschka and the computing center of Johns Hopkins University.

References

d'Albis, A. (1963), *Compt. Rend. 257*, 2341.
d'Albis, A. (1964), *Compt. Rend. 259*, 1779.
d'Albis, A. (1966), *Compt. Rend. 262D*, 574, 1888.
d'Albis, A., and Bechet, J. (1967), *Biochim. Biophys. Acta 140*, 435.
Arrio, B. (1967), *Compt. Rend. 264D*, 1331.
Bender, M. L., Killheffer, J. V., and Roeske, R. W. (1965), *Biochem. Biophys. Res. Commun. 19*, 161.
Bender, M. L., *et al.* (1966), *J. Am. Chem. Soc. 88*, 5890.
Benmouyal, P., and Trowbridge, C. G. (1966), *Arch. Biochem. Biophys. 115*, 67.
Bensusan, H. B., and Nielsen, S. O. (1964), *Biochemistry 3*, 1367.
Blout, E. R., de Loze, C., and Asadourian, A. (1961), *J. Am. Chem. Soc. 83*, 1895.
Bryan, W. P., and Nielsen, S. O. (1960), *Biochim. Biophys. Acta 42*, 552.
Delaage, M., and Lazdunski, M. (1968), *European J. Biochem. 4*, 378.
DiSabato, G., and Ottesen, M. (1965), *Biochemistry 4*, 422.
Feinstein, G., and Feeney, R. E. (1966), *J. Biol. Chem. 241*, 5183.
Glascoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.
Hummel, G. W. (1959), *Can. J. Biochem. Physiol. 37*, 1393.
Hvidt, A. (1963), *Compt. Rend. Trav. Lab. Carlsberg 33*, 475.
Hvidt, A. (1964), *Compt. Rend. Trav. Lab. Carlsberg 34*, 299.
Hvidt, A., and Kagi, J. H. R. (1963), *Compt. Rend. Trav. Lab. Carlsberg 33*, 463.
Hvidt, A., and Nielsen, S. O. (1966), *Advan. Protein Chem. 21*, 287.
Johannin, G., and Yon, J. (1966), *Biochem. Biophys. Res. Commun. 25*, 320.
Kushner, V. P., and Bozhkov, V. M. (1967), *Mol. Biol. 1*, 518.
Lazdunski, M., and Delaage, M. (1965), *Biochim. Biophys. Acta 105*, 541.
Lazdunski, M., and Delaage, M. (1967), *Biochim. Biophys. Acta 140*, 417.
Leichtling, B. H., and Klotz, I. M. (1966), *Biochemistry 5*, 4026.
Linderstrom-Lang, K. (1955), Special Publication 2, London, The Chemical Society.
Luzikov, V. N., and Troshkina, T. V. (1966), *Biofizika 11*, 535.
Mathews, B. W., Sigler, P. B., Henderson, R., and Blow, D. M. (1967), *Nature 214*, 652.
Mikes, O., Tomasek, V., Holeysovsky, V. and Sorm, F. (1967), *Collection Czech. Chem. Commun. 32*, 655.
Miyazawa, T. (1967), *in* Poly-α-Amino acids, Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 69.
Nord, F. F., and Bier, M. (1953), *Biochim. Biophys. Acta 12*, 56.
Oppenheimer, H. L., and Hess, G. P. (1963), *Nature 198*, 689.
Segal, D. M., and Harrington, W. F. (1967), *Biochemistry 6*, 768.
Spande, T. F., Green, N. M., and Witkop, B. (1966), *Biochemistry 5*, 1926.
Steiner, R. F. (1966), *Biochemistry 5*, 1964.
Uralets, V. P., and Luzikov, V. N. (1966), *Biofizika 11*, 699.
Walsh, K. A., and Neurath, H. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 281.
Wilcox, E. (1959), *Biochim. Biophys. Acta 34*, 602.