An Investigation of the Peptic Activation of Acetylated Trypsinogen*

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ABSTRACT: Properties of the enzyme produced by the pepsin-mediated activation of acetylated trypsinogen have been reinvestigated. In the current study, two new azo chromophore-labeled specific inactivators, 4-phenylazodiphenylcarbamyl chloride and fluoride, which rapidly inactivate trypsin and acetylated trypsin on a mole to mole basis, forming yellow derivatives, were used to ascertain the normality of active enzyme in impure preparations and as a chromophoric tag to follow its purification. The active derivative (ATd) was found to differ markedly in its properties from those reported by the earlier workers. It was about the same in molecular size as trypsin (based on comparative gel filtration studies), and had a specific activity *vs.* the trypsin substrate, benzoyl-DL-arginine-*p*-nitroanilide, about six times that of trypsin (per active center). It could be inactivated by 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK), a reagent specific for an essential histidine in trypsin. A partially purified preparation showed isoleucine (or leucine) and valine as N-terminal residues, and an amino acid composition differing relatively little from that of trypsin. It was found that trypsin could activate acetylated trypsinogen at pH 7 to about the same extent as occurred in the pepsin-mediated activation at pH 3.2 or 3.4, provided exhaustive dialysis of the acetylated trypsinogen was first performed to remove traces of the inactivator, diphenylcarbamyl fluoride, that remained following its use in the original acetylation procedure. In some respects, ATd appears to be an acetylated trypsin or a molecule very close to it in composition.

It is now generally accepted that only a small region of any enzyme, the active center, plays a direct role in the exercise of an enzyme's catalytic function. It might follow, therefore, that partial degradation of an enzyme can lead to fragments possessing enzymic activity. Should this be the case, it could be concluded that large portions of biologically active proteins serve little or no biochemical function and are, perhaps, only the vestigial remains of evolutionary development. On the other hand, if portions remote from the "active center" of an enzyme are responsible for determining the proper conformation of the latter, fragmentation would in all likelihood lead to loss of activity.

There have been a number of reports of the production of enzyme fragments possessing enzymic activity. Among the most interesting of these reports is that of Viswanatha and co-workers (Viswanatha *et al.*, 1958; Liener and Viswanatha, 1959; Viswanatha and Liener, 1960) who reported an active fragment of trypsin that could be obtained from acetylated trypsinogen by the action of pepsin. This fragment was described as having a molecular weight of about 6000 (one-fourth that of trypsin), an N-terminal phenylalanine instead of the isoleucine of trypsin, a specific amidase activity about one-third, and an esterase activity about one-half that of trypsin. Especially interesting was their finding of only trace amounts of histidine, the concentration of which was unrelated to the enzymic activity, since there is much evidence linking at least one histidine (of the three in trypsin) to the catalytic mechanism of trypsin (*cf.* Shaw *et al.*, 1965).

There has been considerable controversy concerning these results (Neurath, 1959; Chevallier *et al.*, 1964) particularly with respect to the purity and identity of the material studied. Because the question of the existence of such an active fragment has an important bearing on the problem of the mechanism of action of trypsin, as well as on the general problem of structure–activity relationships, we chose to reinvestigate the problem using experimental techniques capable of establishing unequivocally the properties of the active enzyme as distinguished from contaminant inert protein. For this purpose, two new azo chromophore-"labeled" specific inactivators, 4-phenylazodiphenylcarbamyl chloride (PADPCC)[1] and fluoride (PADPCF) (S. M. Vratsanos,

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[1] Abbreviations used: PADPCC, 4-phenylazodiphenylcarbamyl chloride; PADPCF, 4-phenylazodiphenylcarbamyl fluoride; PADPC, 4-phenylazodiphenylcarbamyl; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; BAPA, benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; ATg, two-times-acetylated trypsinogen; DPCC, diphenylcarbamyl chloride; DPCF, diphenylcarbamyl fluoride; TEPP, tetraethyl pyrophosphate; ATd, active enzyme derivative obtained from pepsin-mediated activation of ATg; TCA, trichloroacetic acid; DMSO, dimethyl sulfoxide; BAEE, α-*N*-benzoyl-L-arginine ethyl ester hydrochloride; TAME, α-*N*-*p*-toluenesulfonyl-L-arginine methyl ester hydrochloride.

H. Kaufman, and B. F. Erlanger, in preparation), have been utilized. They rapidly inactivate trypsin, acetylated trypsin, and chymotrypsin on a mole to mole basis (*cf.* Erlanger *et al.*, 1966), forming yellow derivatives and can be used to ascertain the normality of active enzyme in impure preparations and as a chromophoric tag to follow its purification.

Experimental Section

Materials. Trypsinogen was purchased from Mann Research Laboratories (one-time-crystallized, 50% $MgSO_4$) and Worthington Biochemical Corp. (one-time-crystallized, 92.5% protein). Pepsin (two-times-crystallized) and trypsin (two-times-crystallized) were products of the Worthington Biochemical Corp. Diphenylcarbamyl chloride was a product of Distillation Products, Inc., recrystallized three times from methanol. Diphenylcarbamyl fluoride was prepared from the chloride as described by Metzger and Wilson (1964). 4-Phenylazodiphenylcarbamyl chloride and fluoride were prepared according to the procedure of S. M. Vratsanos, H. Kaufman, and B. F. Erlanger (manuscript in preparation). 1-Chloro-3-tosylamido-7-amino-2-heptanone (TLCK) was obtained as the hydrochloride (A grade) from Calbiochem. Acetyltrypsin was prepared by the procedure of Jansen *et al.* (1951).

Enzyme Assay. Assays were performed with the trypsin substrate, benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA) (Erlanger *et al.*, 1961).

Preparation of the Active Derivative. Twice-acetylated trypsinogen (ATg) preparations were made using the method of Viswanatha *et al.* (1958), except that inactivation of contaminant trypsin was performed with diphenylcarbamyl chloride (DPCC) or fluoride (DPCF) rather than diisopropylfluorophosphate (DFP), and the inactivation was performed after the acetylation of trypsinogen rather than before. (In an initial attempt to inactivate contaminant trypsin using TEPP before acetylation, it was found that the rate of autoactivation was faster than that of inactivation. Prior acetylation of the zymogen eliminated this problem.)

In one preparation, for example, following the second acetylation and dialysis against dilute acetic acid (pH 4 at 5°), DPCC in acetone was added to the two-times-acetylated trypsinogen solution, to give 2000 ml of a 5% acetone solution containing an estimated 5.2×10^{-2} mmole of ATg and 5.2 mmoles of DPCC (not all in solution). The pH was adjusted up to 8.15 and inactivation of contaminant (acetylated) trypsin was allowed to proceed at 25° for 4 hr. After 2 hr and 40 min contaminant tryptic activity as measured by assay with BAPA was reduced from 0.6 to < 0.05% of that of a reference trypsin of the same molar concentration. The preparation was filtered, and after adjusting the pH to 4, it was dialyzed at 5° against dilute acetic acid (pH 4) for 22 hr. It was then lyophilized and used as needed.

In another preparation, DPCF, which inactivates trypsin more rapidly than does DFP or DPCC, was used to inactivate contaminant (acetylated) trypsin. In this case, a tenfold molar excess (based on the amount of trypsinogen) was present during each acetylation. Then the 2500 ml of dialyzed, two-times-acetylated trypsinogen solution (containing 13.2×10^{-2} mmole) was brought to pH 8 and treated in 5% acetone with a tenfold molar excess of DPCF (not all of which went into solution), for about 1.5 hr at 25°. The contaminant tryptic activity was reduced to less than 0.007% of a reference trypsin sample from the 0.034% present just after the second acetylation. After filtering, the solutions were dialyzed against dilute acetic acid (pH 4) at 6° for 22 hr and lyophilized.

The per cent of free amino groups remaining was determined by a quantitative ninhydrin technique (Troll and Cannan, 1953).

Peptic Activation. Activations with pepsin were carried out, as described by Viswanatha *et al.* (1958) and Liener and Viswanatha (1959), at initial pH values of 3.2 and 3.4 for from 48 to about 60 hr at 25° with pH controlled neither by buffer nor pH-Stat. Since the published procedures did not specify whether the pH was maintained constant at the initial value or not, activation was also carried out at pH 3.2, 3.4, and 3.8, kept constant by a Radiometer pH-Stat. Ratios of pepsin to ATg (by weight) varied from 1:24 to 1:94, with ATg concentration varying from 0.87 to 1.1 mg/ml (usually the latter). The time course of activation was studied by assaying aliquots taken as a function of time with the substrate BAPA.

For further studies, the active derivatives (ATd) were precipitated by half saturated $(NH_4)_2SO_4$ at 5°, dialyzed against H_2O at 0° in Visking casing no. 8 until all the $(NH_4)_2SO_4$ was removed, and then lyophilized. Monitoring assays were conducted during dialysis to assure that no significant loss of ATd activity occurred. Removal of $(NH_4)_2SO_4$ was demonstrated by use of the microdiffusion method of Conway (as described in Hawk *et al.*, 1947).

Solubility in 3.6% TCA was performed as described by Viswanatha *et al.*, (1958).

Inactivation of Enzymes by PADPCC and PADPCF. Inactivation of ATd, trypsin, or acetylated trypsin by PADPCC or PADPCF was generally conducted in 50% dimethyl sulfoxide (DMSO), 0.05 M Tris-Cl buffer, 0.02 M $CaCl_2$, "apparent" pH 8.0 at 25°. Use of 50% DMSO as solvent was made because of the low solubility of PADPCC and PADPCF in aqueous solutions. It permitted concentrations of inactivator of 2.4×10^{-4} M to be readily employed. Controls of enzyme in the same solvent were also run. Stock solutions of the inactivator were prepared in acetone. Aliquots were added to the enzyme in 50% DMSO solution to give a reaction solution that was 1% in acetone. When spectral measurements of the solution were desired, methanol rather than acetone was used, since acetone absorbs strongly at 280 mμ. When it was desired to remove excess inactivator from an inactivated enzyme preparation, dialysis in the cold against 0.001 M HCl or gel filtration through a column of Sephadex G-25 suspended in 0.001 M HCl was generally employed.

Determination of the Operational Normality of an ATd Preparation. Two methods were used for this purpose.

In one, the concentration of ATd active centers in an impure preparation was determined by titrating an excess of the ATd preparation (3.56 mg/ml in 50% DMSO, 0.05 M Tris-chloride, 0.02 M $CaCl_2$, apparent pH 8.0) with known concentrations of the azo chromophore-labeled inactivator (PADPCF) (from 0.22 to 1.1 $\times$ 10^{-5} M). Per cent inactivation was measured by assay of the tryptic activity remaining after completion of the reaction. (Four hours was required for all concentrations except 0.22 $\times$ 10^{-5} M, which was complete after 8 hr.) Controls containing enzyme but no inactivator showed no loss of activity. Extrapolation to 100% inactivation of the line obtained by plotting per cent inactivation *vs.* concentration of PADPCF permits determination of the concentration of ATd active centers.

Concentration of active centers was also determined by first treating 2.4 $\times$ 10^{-4} M PADPCF with 6 mg/ml of impure ATd preparation in 50% DMSO, 0.05 M Tris-Cl buffer, 0.02 M $CaCl_2$, 1% acetone, apparent pH 8.0 at 25° for 66 min, by which time 99.95% of the enzyme was inactivated (as compared with a control). Known volumes of the solution were then dialyzed in Visking no. 8 tubing at 6° against 0.001 M HCl to remove DMSO and excess PADPCF. (Minimum time for removal of DMSO was monitored by dialyzing bags of the 50% DMSO solvent under similar conditions, and reading the optical density of dialyzed solution as a function of time. The initial optical density of the 50% DMSO solution at 280 mμ was 1.280.) The dialyzed solutions and washings with 0.001 M HCl were then brought to known volumes with 0.001 M HCl. After clarifying the supernatants by centrifugation in the cold, the optical densities were read on a Beckman Model DU spectrophotometer at 345 mμ, at which wavelength there is a peak absorption of the phenylazodiphenylcarbamyl chromophore linked to ATd (or trypsin) (ϵ_{max} 1.65 $\times$ 10^4 at 345 mμ).

Gel Filtrations. ESTIMATION OF MOLECULAR SIZE. Gel filtrations of ATd were performed using Bio-Gel P-10 and Sephadex G-50 and G-75 columns equilibrated in aqueous solutions of low (0.001 M HCl) as well as relatively high ionic strengths (0.05 M glycine-HCl, and $CaCl_2$ or 0.1 M KCl–0.001 M HCl) at pH 3.0 either at room temperature (about 25°) or at 6–8°. For comparative purposes, gel filtrations of trypsin through the same columns and under the same conditions were also run.

The gel bed was stabilized by eluting at a rate of 6–7 ml/cm^2 per hr for a time sufficient to permit a volume of eluent at least three times that of the gel bed to pass through. Subsequent to this, a sample of 0.2% Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.), which is a colored high molecular weight dextran, was passed through the column to check on homogeneity of the gel bed and to determine its void volume. The ratio of volume of sample (containing enzyme) to bed volume was at least 1:22, and was 1:100 for runs through Sephadex G-75. Fractions of 1 ml were collected. Flow rates were from 6 to 7 ml/cm^2 per hr.

Aliquots of fractions were assayed with BAPA. The elution volume, V_e, corresponding to maximum enzyme activity was estimated to the nearest milliliter from an elution diagram.

Preparative Gel Filtration. Various sized columns of Sephadex G-25 and G-75 were employed for the purification of 4-phenylazodiphenylcarbamyl derivatives of ATd. Fractions were monitored at 280 (to follow protein) and 345 mμ (to follow the PADPC chromophore) by reading their optical densities on a Beckman Model DU spectrophotometer and recording the ultraviolet spectra of selected fractions on a Cary Model 11 M recording spectrophotometer.

In one case (see below), a Sephadex G-75 column, suspended in 0.001 M HCl at 8–10°, that was 1.9 cm in internal diameter and 73 cm in height (213-ml bed volume), was used to further purify a 4-ml sample containing 75 mg of a lyophilized preparation of PADPC-ATd. A flow rate of 7 ml/cm^2 per hr was maintained, and 3-ml fractions were collected. The same column was used for a second gel filtration of the lyophilized material from the peak fractions having the highest $OD_{345m\mu}$:$OD_{280m\mu}$ ratios. The peak fractions obtained from this second run were then lyophilized and used for further studies.

N-Terminal group analyses were performed using 2,4-dinitrofluorobenzene (Fraenkel-Conrat *et al.*, 1955; Levy, 1954).

Amino acid analysis of the most purified PADPC-ATd preparation was carried out with the use of a Technicon amino acid analyzer.

Reactions with TLCK. Inactivation experiments between ATd or trypsin and TLCK, with the latter in substantial excess (in a molar ratio of at least 50:1), were carried out in 0.05 M Tris-HCl–0.05 M $CaCl_2$ (pH 7.0) at 25°. In one experiment, for example, 3 $\times$ 10^{-4} M TLCK was treated with preparation A (1.5 mg of ATd preparation/ml, 0.55 $\times$ 10^{-5} M in active centers) under these conditions for 3 hr. Controls in which TLCK was absent were run in otherwise similar conditions.

Results

Acetylation of Trypsinogen. Between 82 and 90% of the free amino groups of trypsinogen was acetylated, depending on the batch. Viswanatha *et al.* (1958) had obtained 87% acetylation.

Peptic Activation of Acetylated Trypsinogen. It was our purpose to reproduce the activation conditions of the earlier workers as closely as possible in order to eliminate the possibility of obtaining a product other than the reported one. However, two different sets of conditions were described. In one (Viswanatha *et al.*, 1958), peptic digestion was carried out at 25° and pH 3.4, with a ratio of zymogen to pepsin of from 20:1 to 60:1. Presumably, the resulting product was the same regardless of the ratio used. In a second paper (Liener and Viswanatha, 1959), the peptic digestion was done at 25° and pH 3.2, with an initial ratio of zymogen to pepsin of 94:1. In neither of the papers was there any information as to whether the pH was maintained constant and, if so, whether by buffering or by use of a pH-Stat. In the former paper (Viswanatha *et al.*, 1958), at a

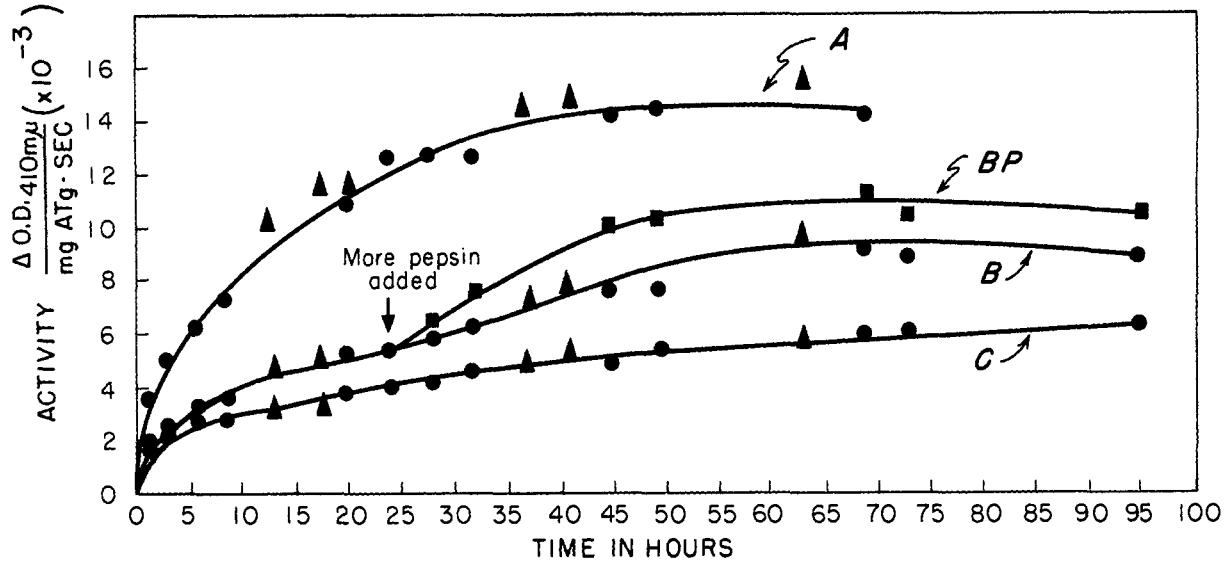

FIGURE 1: Time course of activation of acetylated trypsinogen (ATg) treated with pepsin; pH *not* maintained constant. Concentration of ATg, 1.1 mg/ml; temperature, 25°. Curve A: initial pH 3.4; ratio of pepsin to ATg was 1:24 (w/w). Curve B: initial pH 3.2; ratio of pepsin to ATg was 1:50 (w/w). Curve BP: conditions initially were the same as for curve B, but, after 24 hr, fresh pepsin was added in an amount equal to that present initially, so that the ratio of pepsin to ATg then became 1:25 (w/w). Curve C: initial pH 3.0; ratio of pepsin to ATg was 1:50 (w/w). Aliquots were taken at intervals for assay with BAPA substrate. Activity of a reference commercial two-times-crystallized trypsin expressed in units of $\Delta OD_{410\ m\mu}$/sec per mg of trypsin was 27.6×10^{-3}.

zymogen to pepsin ratio of 24:1, maximal activity was found after 6 hr of digestion, at which time solubility in TCA was about 50%. After 18 hr, TCA solubility increased to 80%, the activity dropping to about 85% of its earlier maximum. In the latter case (Liener and Viswanatha, 1959), at a zymogen to pepsin ratio of 94:1, maximal activity appeared between 12 and 24 hr. TCA solubility was 84% after 24 hr. After the addition of more pepsin, digestion was continued for 59 hr. Apparently most of the subsequent purification and characterization studies were carried out on this material.

From the above, it is apparent that no single activation procedure would suffice for our studies. The problem of pH (whether it was kept constant or not) was particularly important. Therefore, we chose to vary the conditions within the framework of the data of Viswanatha and his colleagues.

The amount of tryptic activity obtained under the various conditions (all assays were made with BAPA) varied from about 18 to 55% of that of a comparable weight per volume of commercial trypsin. (The earlier reports indicated an activity of about 20% of that of trypsin, using BAEE as substrate.) Higher final activities were obtained, in general, as the pH was increased (see Figures 1 and 2). Thus, in a series of activations performed at initial pH values of 3.0, 3.2, and 3.4 in which no effort was made to keep pH constant (see Figure 1), the lowest final activity obtained was for the case in which initial pH was 3.0 (curve C) and the highest when the initial pH was 3.4 (curve A). In the latter case, a higher pepsin to ATg ratio (1:24, w/w) was utilized than for the activations at an initial pH of 3.0 (curve C) or 3.2 (curve B) for which the ratios were 1:50. However, even when more pepsin was added to part of the solution that was initially at pH 3.2 (see curve BP), making the ratio almost the same as that in the solution represented by curve A, the final level of activity still did not attain the level of the latter. As expected, in all four cases as the time of hydrolysis increased, the pH also increased, becoming after 65 hr 3.6 for C, 3.9 for B, and 4.3 for A.

When the pH was maintained constant using a pH-Stat (see Figure 2), less activity was obtained but the maximum was reached earlier than when the pH was not controlled. (Curve A of Figure 1 is reproduced for comparison.) It is also to be noted that at a constant pH of 3.8, a higher peak activity was achieved than at pH 3.4, and that in both cases a significant fall-off in activity with time occurred after the peak was reached.

An activation experiment at pH 3.2 was carried out under conditions closely paralleling those reported by Liener and Viswanatha (1959) as being used in the preparation of ATd for further studies, except that in our experiment the pH was maintained constant using a pH-Stat. The concentrations were 0.87 mg of ATg/ml and 0.0093 mg of pepsin/ml, to which an additional 0.0061 mg of pepsin/ml was added after 24 hr. The pattern of activation, both in terms of tryptic activity and in solubilization of the protein in 3.6% TCA, was very similar, particularly during the first 24 hr, although, after 59 hr, the TCA solubility which reached 100% in their experiment only went up to 92% in ours. In spite

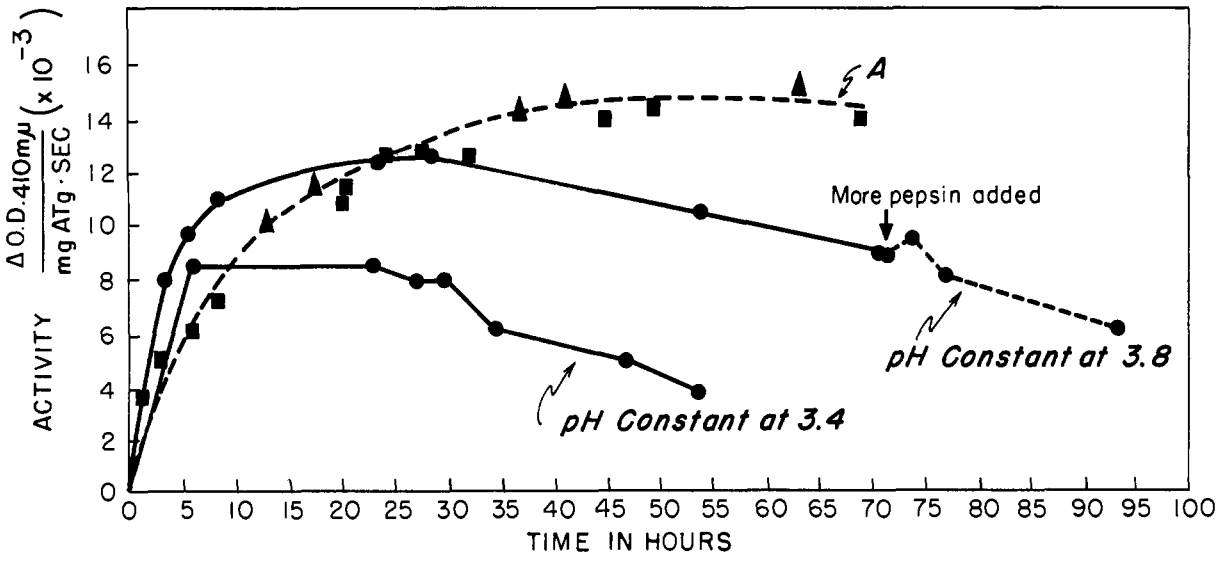

FIGURE 2: Time course of activation of acetylated trypsinogen treated with pepsin; *pH maintained constant* by pH-Stat (solid lines). Curve A (dotted line): as in Figure 1 (pH *not* maintained constant, initial pH 3.4); shown for purposes of comparison. Concentration of ATg, in all cases shown, 1.1 mg/ml; that of pepsin was 0.0465 mg/ml. Activity of a reference commercial two-times-crystallized trypsin expressed in units of $\Delta OD_{410\ m\mu}$/sec per mg of trypsin was 27.6×10^{-3}.

of the difference between the activation pattern obtained under these conditions and that shown for curve A in Figure 1, the molecular size and other important characteristics of the ATd produced in both instances was similar, as will be brought out subsequently.

Preparations of ATd Used for Further Study. ATd prepared by the procedure mentioned just above, *i.e.*, at pH 3.2, maintained constant by pH-Stat, etc., will be referred to as preparation L. ATd obtained by activation (for 46 hr) of ATg by pepsin under the conditions described for curve A in Figure 1 (*i.e.*, at initial pH 3.4, with pH not maintained constant), which was then fractionated with half-saturated $(NH_4)_2SO_4$ and subsequently dialyzed and lyophilized, will be referred to as preparation A. A sample of ATd so prepared had about 80% the activity against BAPA as the same weight of a reference commercial two-times-crystallized trypsin.

Estimation of Molecular Size of ATd by Gel Filtration. Of signal interest in the characterization of ATd is the question of its molecular weight which was reported to be about one-fourth that of trypsin by Viswanatha and Liener (1960). The gel filtration technique provides an indication of the molecular size of molecules, and correlations between elution volume, V_e, or distribution coefficient, K_D, and molecular weight of proteins have been expressed (Andrews, 1964).

In all gel filtrations that were run, the elution volumes for ATd were almost identical with those required for elution of trypsin in the same columns (see Table I). This was true whether the eluent at pH 3 was of relatively high ionic strength, such as 0.1 M KCl, or low ionic strength (0.001 M HCl). Nor did it matter, in this respect, whether preparation A or L was used. Thus, in an early run with Bio-Gel P-10, a polyacrylamide gel which is designed to exclude molecules of above 10,000 in molecular weight, the elution volume for the former was 15.5 ml and for trypsin, 17.5 ml. This indicated, at the least, that the ATd was of a molecular weight greater

TABLE 1: Gel Filtration Studies.[a]

Gel	Eluent	ATd Prepn	Elution Vol. (ml) ATd	Elution Vol. (ml) Trypsin
Bio-Gel P-10[b]	0.001 M HCl	A	15.5	17.5
Sephadex G-75[c]	0.05 M glycine-HCl–0.05 M $CaCl_2$ (pH 3.0)	A	28.0	30.0
Sephadex G-75[d]	0.1 M KCl–0.001 M HCl (pH 3.0)	L	23.6	23.0

[a] In all runs, sample volume was 0.5 ml; volume of fractions collected, 1 ml; flow rate, 6–7 ml/cm² per hr. [b] Column dimensions, 1.0 × 38.3 cm; bed volume, 30 ml; and temperature, 25°. [c] Column dimensions, 1.1 × 55 cm; bed volume, 52 ml; void volume, 18 ml; and temperature, 8°. [d] Column dimensions, 1.5 × 28 cm; bed volume, 50 ml; void volume, 15.7 ml; and temperature, 6°.

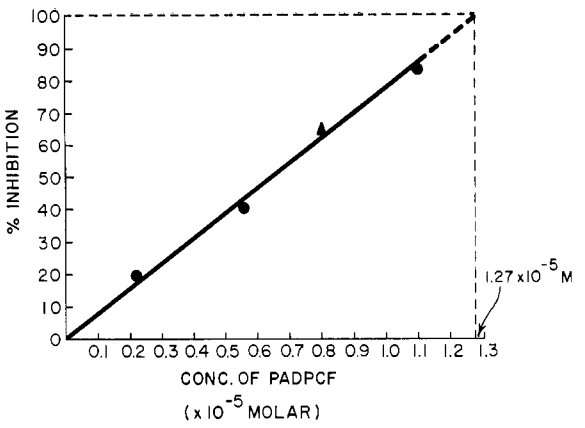

FIGURE 3: Determination of enzymic operational molarity. Reaction conditions: 3.56 mg of ATd/ml was treated with PADPCF.

than 10,000, and that its molecular size was probably not significantly smaller than that of trypsin.

Using a column of Sephadex G-75 (second line of Table I) the elution volume of preparation A was 28 ml; that of a sample of trypsin passed through the same column was 30 ml. When gel filtration of preparation L was performed through a column of Sephadex G-75 (third line of Table I), the elution volume was 23.6 ml. The elution volume for a sample of trypsin that was gel filtered under the same conditions was 23 ml. Thus, both preparations of ATd behave as if their molecular weights are the same as that of trypsin, *i.e.*, *ca.* 25,000. As a further check, it was calculated from Andrews' work (Andrews, 1964) that a protein with a molecular weight of 6000 would have had an expected elution volume of about 36 ml under the conditions of gel filtration used for preparation L. The actual elution volume found (*i.e.*, 23.6 ml) is, thus, well beyond the experimental error.

Use of the Chromophoric Fluoride, PADPCF, to Determine Operational Normality of an Impure ATd Preparation and Its Specific Molar Activity. The key issue of the controversy concerns the purity and homogeneity of the preparations of the earlier workers. One cannot, for example, relate the concentration of histidine to activity if the molar concentration of active enzyme is not known. Nor can the activation process be understood without knowledge of the efficiency of the process, *i.e.*, the number of moles of active material produced per mole of acetylated trypsinogen. This aspect of the problem was investigated by titrating the active centers of preparation A, using the specific inactivator PADPCF for this purpose. Figure 3 shows the results of such a titration in which known concentrations of PADPCF were treated with an excess of ATd and per cent inactivation was then measured by assay of the tryptic activity. (ATd controls lost no activity under the same conditions.)

Extrapolation of the line to 100% inactivation shows that this preparation A was 1.27×10^{-5} M in active centers. Furthermore, knowing its tryptic activity, we could ascertain that ATd is about *six times* as active on a molar basis against BAPA as a reference sample of trypsin. The linearity of the curve indicates that the ATd molecules are of a single species so far as activity per active center is concerned.

Confirmation of the concentration of ATd active centers was obtained by measuring the absorbance of a dialyzed preparation of ATd completely inactivated by PADPCF. Using the molar absorbance of the azo chromophore (ϵ_{max} 1.65×10^4 at 345 mμ), the molar concentration of ATd active centers was calculated and found to agree closely with the value in Figure 3.

It should be pointed out that the high specific activity of ATd, namely six times that of trypsin, indicates that *only about 5–10% of the original acetylated trypsinogen was finally activated to ATd.*

Inactivation of ATd by TLCK. TLCK, a reagent that inactivates trypsin by reacting specifically with an essential histidine (Shaw *et al.*, 1965), was also found to inactivate ATd. When TLCK (3×10^{-4} M) was treated with preparation A (1.5 mg of ATd preparation/ml, 0.55×10^{-5} M in active centers), 98% inactivation occurred in 2 hr. (A tryptic preparation of slightly higher concentration in active centers was completely inactivated in the same time.) Preparation L was similarly inactivated by TLCK.

Amino Acid Composition and N-Terminal Amino Acid

TABLE II: Amino Acid Composition of P-ATD$_{II-p}$ Compared with That of Trypsin.

Amino Acid	Trypsin[a]	P-ATD$_{II-p}$[b]
Aspartic acid (asparagine)	22	22
Threonine	10	10
Serine	33	22
Glutamic acid (glutamine)	14	14
Proline	9	9
Glycine	25	22
Alanine	14	14
Half-cystine	12	12
Valine	17	15
Methionine	2	2
Isoleucine	15	14
Leucine	14	14
Tyrosine	10	8
Phenylalanine	3	3
Histidine	3	3
Lysine	14	14
Tryptophan	4	
Arginine	2	2

[a] Based on composition of trypsinogen according to Walsh and Neurath (1964). [b] 4-Phenylazodiphenylcarbamyl-ATD$_{II-p}$ based on 2 moles of arginine/mole of P-ATd$_{II-p}$.

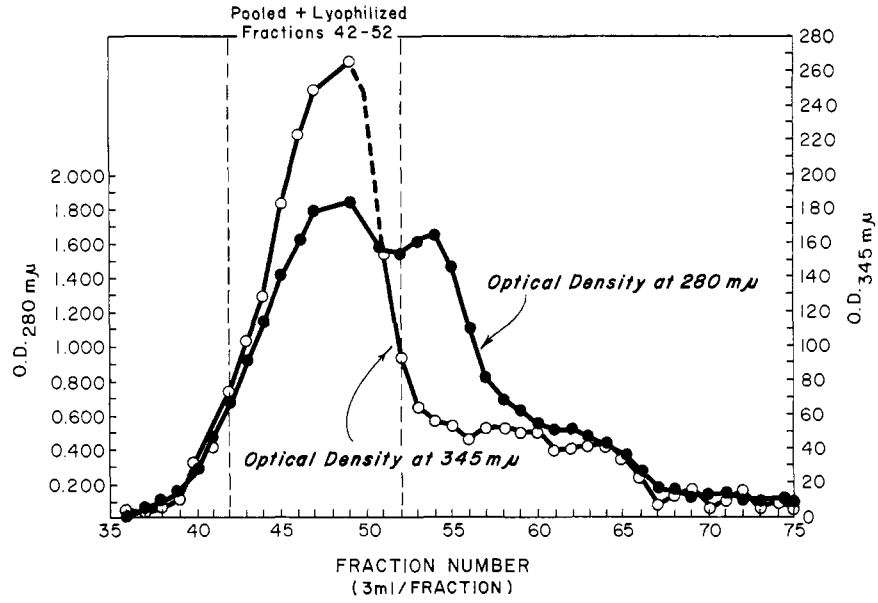

FIGURE 4: First gel filtration of PADPC-ATd through Sephadex G-75. Sephadex G-75 was in 10^{-3} M HCl at 9°. The column was 1.9 × 73 cm; flow rate, 7 ml/cm² per hr; sample, 4 ml. Absorbance at 345 mμ was monitored to follow the presence of the PADPC chromophore.

Determination of a Sephadex G-75 Gel-Filtered Preparation of PADPC-ATd. To characterize the active derivative (ATd) further, as well as to gain understanding of the mechanism of activation of acetylated trypsinogen, it would, of course, be useful to have purified ATD. In pursuit of this objective, preparation A, which, as noted earlier, was already partially purified by $(NH_4)_2SO_4$ fractionation, was further purified, after inactivation with an 11 molar excess of PADPCF and dialysis to remove excess reagent, by chromatographing through Sephadex G-75. The fractions containing the azo chromophore-labeled ATd were determined by monitoring at 345 and 280 mμ. A fairly sharp peak of 345-mμ absorbing fractions can be seen in the elution diagram (Figure 4). Fractions from this peak containing 71% of the total 345-mμ absorbing material were rechromatographed on Sephadex G-75, yielding the results shown in Figure 5.

Fractions 43′ to 46′, which had the highest ratio of 345:280-mμ absorption, namely 1:7, and therefore considered purest in labeled ATd, were lyophilized. The ratio of the absorption at 345:280 mμ of a similarly labeled trypsin would be about 2.5:7, suggesting that this "most purified" inactivated enzyme preparation contained some denatured, unreactive ATd, or some acetylated trypsinogen that became neither activated nor degraded in the original activation step.

N-Terminal group analyses yielded equal amounts of valine and isoleucine (or leucine). No N-terminal phenylalanine, as ascribed to the active derivative studied by Viswanatha and Liener (1960), could be found.

The amino acid composition of this material (called P-ATD_{II-p}) compared with that of trypsin is shown in Table II. It can be seen that the composition, with the exception of serine, is similar to that of trypsin, which is consistent with the previous observations on molecular size determined by gel filtration. It is also to be noted that all the three histidines found in trypsin are present.

Discussion

Using techniques that permit a more reliable assignment of properties to the active enzyme as distinguished from contaminant inert protein, it has been found that the active enzyme, ATd, obtained by the pepsin-mediated activation of acetylated trypsinogen differs substantially in its properties from those ascribed to it by Viswanatha and Liener (1960). In particular, two of the most interesting characteristics of ATd have not been confirmed: its low molecular weight (about one-fourth that of trypsin), and its lack of a functional histidine residue. The latter, as noted earlier, was of particular interest since there is much evidence linking at least one of the three histidines in trypsin to its catalytic mechanism. Our study indicates that ATd has a molecular weight similar to that of trypsin and that histidine is present and necessary to the activity of the enzyme. Furthermore, our ATd appears to have a specific amidase activity about six times, rather than three-tenths times, that of trypsin, and is not N terminal in phenylalanine as was said to be the case for the ATd described by the earlier workers. In a number of respects, on the basis of the studies reported here, ATd appears to be an acetylated trypsin or a molecule very close to it in composition.

Every effort was made to reproduce the activation

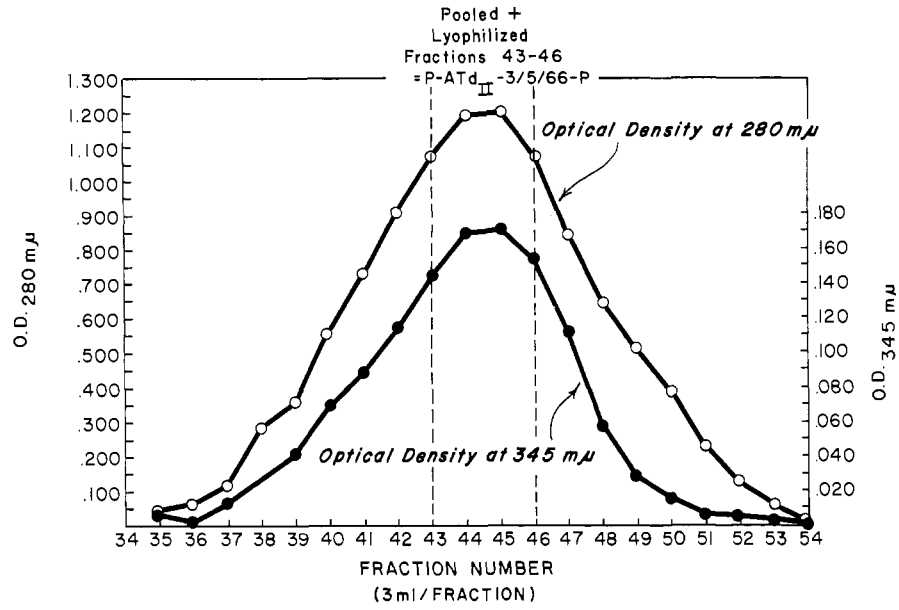

FIGURE 5: Second gel filtration. The material, containing PADPC-ATd, from the peak fractions 42–52 of the first gel filtration, was gel filtered through the same column and under the same conditions as were used for the first gel filtration (see Figure 4); sample, 1.5 ml.

conditions of Viswanatha and his colleagues as closely as possible, and because of some ambiguity in this regard, ATd was prepared by two different procedures. Both preparations were similar in molecular size as determined by gel filtration and both were inactivated by TLCK, the latter finding suggesting the presence of an essential histidine. Furthermore, at each stage in the preparation of ATd to be used for further study, such as in the $(NH_4)_2SO_4$ fractionation and subsequent dialysis,[2] care was taken that there should be no significant loss in active material. We believe, on the basis of the foregoing, that the active enzyme in our ATd preparation was in all likelihood the same as that in the preparations studied by Viswanatha and colleagues.

We consider it possible that the discrepancies between the characterization of ATd by the previous workers, and that by us, may reflect the presence of a substantial amount of inert protein in their "purified" preparation. In this regard, two points should be noted. First of all, the high specific activity of our ATd indicates that only a small fraction of acetylated trypsinogen ultimately becomes active ATd. Thus, since the maximal activity obtained from pepsin-mediated activation of acetylated trypsinogen varied from 18 to 55% of that of a comparable weight of commercial trypsin (Viswanatha and his colleagues found about 20%) and the specific molar activity of ATd is six times that of trypsin, only 3 to 9% of the original acetylated trypsinogen was ultimately converted to active ATd. Second, if one adjusts upward their low ATd specific amidase activity of three-tenths times to our higher one of six times that of trypsin, their low histidine content (from 0.15 to 0.46 histidine/mole of ATd) correspondingly approaches a value consistent with that of an acetylated trypsin derivative and with our analytical findings (Table II). Finally, it should be noted that it is difficult to reconcile the amino acid composition and N-terminal phenylalanine residue ascribed to ATd by Viswanatha and Liener with any portion of the now known primary structure of trypsinogen (Walsh and Neurath, 1964; Mikeš *et al.*, 1966).

If one accepts the conclusions cited above, questions that remain concern the identity of ATd and the mechanism by which it is produced by peptic activation of acetyltrypsinogen. These questions can best be answered by a study of a highly purified ATd preparation. We are presently engaged in carrying out this further purification. On the other hand, a number of conclusions can be drawn from the information at hand and from some preliminary experiments already completed. As stated earlier, ATd is similar to an acetylated trypsin in a number of ways: in molecular size as shown by gel filtration studies, in amino acid composition (Table II), in specific activity (Trenholm *et al.* (1966) found that acetylated trypsin had an activity four times that of trypsin against TAME), and in N-terminal amino acid. With respect to the last property, we cannot assign the N-terminal isoleucine to the active enzyme with certainty at this stage of purification, but the results are consistent with our most purified preparation being a mixture of ATd and

[2] We find that ATd does not pass through the dialysis membrane to a significant degree. In one paper (Viswanatha *et al.*, 1958), losses as great as 40% were reported to occur in dialysis. Later, however, Viswanatha and Liener (1959) employed dialysis in their purification procedure without any note of loss of material.

undegraded acetylated trypsinogen, which has an N-terminal valine residue.[3]

With respect to the mechanism by which ATd is produced, it is important to note that the fraction of acetyltrypsinogen ultimately converted to active enzyme is similar to the fraction of the amino groups of trypsinogen left unacetylated by acetic anhydride. It is possible, therefore, that ATd is produced only from those molecules having an unacetylated ϵ-amino group on the lysine residue of the critical Lys-Ile bond. In this respect, there is a report by Pechère and Neurath (1957) that an acetylated trypsinogen preparation, in which 89% of the free amino groups were acetylated, could be activated by trypsin to an extent compatible with the presence of about 14% of acetyltrypsinogen in which the critical lysine residue was unacetylated. Viswanatha and his colleagues reported that their preparations of acetyltrypsinogen could not be activated by trypsin. We found the same to be true for our preparations. On the other hand, in following their procedure, a large excess of trypsin inactivator was added during the acetylation process (DPCF by us; DFP by Viswanatha *et al.*, 1958). The possibility arose that some inactivator still remained after the processing of the acetylated trypsinogen and that this inactivator prevented activation by added trypsin (which is present at a ratio to trypsinogen of only 1:100). To test this possibility, one of our preparations was exhaustively dialyzed to remove residual DPCF. It could now be activated by trypsin to an extent similar to that of the preparation of Pechère and Neurath (1957). The dialysis procedure used by Viswanatha and his colleagues is not presented in sufficient detail to be sure that they, too, had trypsin inactivator present in their isolated acetyltrypsinogen. On the other hand, we consider it to be a likely possibility since a 50-fold excess of DFP over trypsinogen was added initially. This amounts to a 5000-fold excess over the quantity of trypsin ultimately used in the attempt to activate acetyltrypsinogen (Viswanatha *et al.*, 1958). The difficulty in removing excess DFP from trypsin after dialysis "with repeated changes of water" followed by lyophilization is documented by earlier studies of Viswanatha (1957).

The crucial question remaining is that concerned with the role of pepsin in the activation of acetyltrypsinogen. We have only begun to examine this question. That pepsin does play a role in the activation process is certainly implied by the results of an experiment in which a control solution of acetylated trypsinogen lacking pepsin was run under conditions otherwise the same as those used for a pepsin-mediated activation. A small rise in contaminant tryptic activity was observed, but after 46 hr the activity was only about 2% of that obtained in the presence of pepsin. Furthermore, when pepsin was added to the control after the 46 hr, a rapid rise in tryptic activity was observed. Pepsin may play a direct role in the activation of some molecules either by cleaving the usual Lys-Ile bond involved in the activation of trypsinogen or, for example, the bond between what ordinarily becomes the N-terminal isoleucine of trypsin and an adjacent valine. We are continuing our investigation into the mechanism by which pepsin treatment of acetylated trypsinogen results in active enzyme.

Our studies indicate that the activation of acetylated trypsinogen does not lead to the production of a small fragment of trypsin possessing enzymic activity. Further, the existence of another dialyzable active fragment of trypsin reported by Bresler *et al.* (1962) to be produced in high yield following trypsin autolysis has not been borne out by subsequent experiments. Using what we considered to be a more appropriate assay system, we were unable to duplicate their results, finding only a trace (0.15%) of dialyzable active material (H. Kaufman and B. F. Erlanger, unpublished results). More recently, Bresler and co-workers (1966) reported that gel filtration and other procedures revealed no active low molecular weight fragments of trypsin following autolysis. Earlier reports of an active fragment of papain (Hill and Smith, 1960) have recently been reexamined and found to be inaccurate (Frater *et al.*, 1965). Reports of an active low molecular weight fragment of pepsin are in the literature (Perlmann, 1954; Tokuyasu and Funatsu, 1962) but certain experiments, similar to our own, distinguishing active from inert material, have so far as we know not yet been run. At the moment, it appears that an early optimism with respect to the production of active enzyme fragments may not have been warranted, and that the activity of an enzyme is more dependent upon the integrity of parts of the protein molecule distant (with respect to primary structure) from the active center than was originally envisaged.

[3] There are other possible explanations for the results of the N-terminal amino acid analysis. (a) ATd is N terminal in both valine and isoleucine, but owing to partial denaturation during the isolation procedure, only one-half of it is in the active form, capable of reacting with PADPCF. (b) There are two active trypsin species, one an acetylated trypsin and the other lacking the N-terminal isoleucine, having instead an N-terminal valine, which is the penultimate residue in trypsin. One would further have to propose that partial denaturation has occurred. The true reason for the results will be apparent only when ATd is purified further.

References

Andrews, P. (1964), *Biochem. J. 91*, 222.

Bresler, S., Champagne, M., and Frenkel, S. (1962), *J. Chim. Phys. 59*, 180.

Bresler, S. E., Krutjakov, V. M., and Popov, A. G. (1966), *Biokhimiya 31*, 776.

Chevallier, J., Jacquot-Armand, Y., and Yon, J. (1964), *Biochim. Biophys. Acta 92*, 521.

Erlanger, B. F., Cooper, A. G., and Cohen, W. (1966), *Biochemistry 5*, 190.

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys. 95*, 271.

Fraenkel-Conrat, H., Harris, J. L., and Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 359.

Frater, R., Light, A., and Smith, E. L. (1965), *J. Biol. Chem. 240*, 253.

Hawk, P. B., Oser, B. L., and Summerson, W. H. (1947), Practical Physiological Chemistry, 12th ed, Philadelphia, Pa., Blakiston, p 826.

Hill, R. L., and Smith, E. L. (1960), *J. Biol. Chem. 235*, 2332.
Jansen, E. F., Curl, A. L., and Balls, A. K. (1951), *J. Biol. Chem. 189*, 671.
Levy, A. L. (1954), *Nature 174*, 126.
Liener, I. E., and Viswanatha, T. (1959), *Biochim. Biophys. Acta 36*, 250.
Metzger, H. P., and Wilson, I. B. (1964), *Biochemistry 3*, 926.
Mikeš, O., Tomášek, V., Holeyšovský, V., and Šorm, F. (1966), *Biochim. Biophys. Acta 117*, 281.
Neurath, H. (1959), *J. Cellular Comp. Physiol. 54, Suppl. 1*, 193.
Pechère, J. F., and Neurath, H. (1957), *J. Biol. Chem. 229*, 389.
Perlmann, G. E. (1954), *Nature 173*, 406.
Shaw, E., Mares-Guia, M., and Cohen, W. (1965) *Biochemistry 4*, 2219.
Tokuyasu, K., and Funatsu, M. (1962), *J. Biochem. (Tokyo) 52*, 103.
Trenholm, H. L., Spomer, W. E., Wootton, J. F. (1966), *J. Am. Chem. Soc. 88*, 4281.
Troll, W., and Cannan, R. K. (1953), *J. Biol. Chem. 200*, 803.
Viswanatha, T. (1957), *Compt. Rend. Trav. Lab. Carlsberg 30*, 183.
Viswanatha, T., and Liener, I. E. (1960), *Biochim. Biophys. Acta 37*, 389.
Viswanatha, T., Wong, R. C., and Liener, I. E. (1958), *Biochim. Biophys. Acta 29*, 174.
Walsh, K. A., and Neurath, H. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 884.

Exposure of Tyrosine Residues in Protein. Reaction of Cyanuric Fluoride with Ribonuclease, α-Lactalbumin, and β-Lactoglobulin*

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ABSTRACT: The state of tyrosine residues (accessible or buried) in ribonuclease, α-lactalbumin, and β-lactoglobulin has been examined with cyanuric fluoride. Ribonuclease was found to contain three reactive and three unreactive residues, the reactive ones being of different degrees of reactivity. α-Lactalbumin contains four reactive residues and one unreactive residue. The reactive residues are of two types, three being of the same degree of reactivity, the fourth being less reactive. β-Lactoglobulin contains three reactive residues and one unreactive residue. The reactive ones can be subdivided into two residues of greater and one of lower reactivity.

Anomalous tyrosine ionization behavior in proteins was first observed in ovalbumin by Crammer and Neuberger (1943), who concluded that tyrosine residues in ovalbumin are not free to ionize in the native state due to restrictions imposed upon the protein configuration by its tertiary structure. Since then it has become generally accepted to regard the idiosyncrasies of tyrosine behavior in proteins as reflections of the secondary and tertiary structures of the proteins (Beaven, 1961; Wetlaufer, 1962). The forces which prevent ionization of tyrosine residues in native proteins are believed to be either hydrogen bonds to specific acceptor groups, as first suggested by Crammer and Neuberger (1943), or hydrophobic forces which cause uncharged tyrosine side chains to be buried in the protein interior, as proposed by many authors (Yanari and Bovey, 1960; Williams and Foster, 1959; Tanford, 1962), or a combination of the two effects (Edsall, 1963).

It is customary to divide the tyrosine residues of proteins into two broad classes, normal and buried. Normal tyrosine residues are very close to simple tyrosine peptides in their ionization behavior; they are assumed to be completely exposed to solvent molecules. Buried tyrosine residues do not resemble simple tyrosine peptides in their ionization behavior and are assumed to be prevented from intimate contact with solvent molecules (Beaven, 1961; Wetlaufer, 1962; Edsall, 1963).

In reality, a rigorous classification into completely exposed and completely buried residues is a great oversimplification, because frequently one is dealing with residues of an intermediate class, neither fully exposed nor fully buried. This fact has been stressed repeatedly by Laskowski (1966) and elegantly demonstrated for the case of tryptophan exposure in α-chymotrypsinogen and lysozyme (Williams and Laskowski, 1965; Williams *et al.*, 1965).

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