parameter which is responsible for the pH shifts observed for the clot opacity, syneresis, and breaking weights.

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Bovine Fibrinogen. II. Effects of Tyrosine Modification on Fibrin Monomer Aggregation†

H. M. Phillips and J. Lyndal York*

ABSTRACT: Contrary to earlier reports of 35–40 free tyrosines we have found 15–20 tyrosine residues exposed in fibrinogen. No additional residues are exposed on conversion of fibrinogen to fibrin, indicating no large conformational change as a result of this conversion. In addition it is shown that modification of tyrosine does not change the clotting time and clottability of fibrinogen, but does decrease the clot strength. The evidence indicates that only two to three modified tyrosine residues are responsible for the change in clot strength. Since modification of these tyrosine residues does not inhibit clotting but changes the physical properties of the clot formed, tyrosine hydrogen bonding is not the driving force for aggregation but rather may serve in the alignment of the fibrin monomers preceeding aggregation.

Studies by several investigators leading to the suggestion that lysine and/or tyrosine may play a role in fibrin polymerization have been reviewed (Phillips and York, 1973). If tyrosine residues are involved in fibrin polymerization they must certainly be exposed in fibrin and perhaps in fibrinogen but not necessarily so. Thus the determination of the number of available tyrosine residues in fibrinogen and fibrin and the effect of tyrosine modification on clottability and the physical properties of the resulting clots is meaningful in understanding fibrin polymerization.

Aside from the possible role of tyrosine in fibrin polymerization, a knowledge of the number of accessible tyrosine and lysine residues as well as other functional groups is evidence which would be useful in distinguishing between various models that have been suggested for the structure of fibrin. For instance, the model of three globular units connected by thin rods proposed by Hall and Slayter (1959) suggests a more compact structure than the "birdcage" of Köppel (1970) which predicts 90% of the molecule is exposed to solvent.

Previous attempts to determine the number of accessible tyrosine residues in fibrinogen by acylation with *N*-acetylimidazole (Huseby and Murray, 1967, 1969) or by spectrophotometric titration (Mihalyi, 1968) yielded values in the range of 35–40.

Chemical modification of amino acids on proteins has long been used to distinguish between freely available and buried residues; however, this approach may yield anomalous results if the reagent has low specificity and reaction occurs with another residue such as lysine resulting in a conformational change in the protein and the exposure of a normally buried residue such as tyrosine. Likewise, spectrophotometric titration will give high values if the protein conformation is sensitive to pH as is the case with fibrinogen (Mihalyi, 1965).

To circumvent these problems we wish to report the number of free tyrosine residues in fibrinogen using a tyrosine-specific reagent, tetranitromethane (Sokolovsky *et al.*, 1966), and in amidinated fibrinogen and amidinated fibrin using *N*-acetylimidazole. Data are also presented on the breaking weight, a measure of clot strength, and clottability of tyrosine-modified fibrin clots.

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Materials and Methods

The preparation of bovine fibrinogen, bovine thrombin, and 84% amidinated fibrinogen has been described (Phillips and York, 1973).

Fibrinogen and 84% amidinated fibrinogen were acetylated with *N*-acetylimidazole (Eastman) at a protein concentration of 8–10 mg/ml in 0.02 M barbital–0.15 M NaCl (pH 7.5), 25° with 5- and 20-fold molar excess of reagent per mole of tyrosine and lysine and 5-fold molar excess per mole of tyrosine for fibrinogen and amidinated fibrinogen, respectively. The *N*-acetylimidazole was first dissolved in a small volume of 0.04 M sodium barbital (pH 7.5). Aliquots were removed at the time indicated and passed through G-25 columns equilibrated with 0.02 M sodium barbital–0.15 M NaCl (pH 7.5) or dialyzed at 0° if protein became viscous or insoluble. Amidinated fibrinogen was also acetylated as described above in urea of varying molar concentrations for 1 hr at which time the samples were dialyzed against urea of the same concentration in 0.02 M sodium barbital–0.15 M NaCl (pH 7.5). For larger quantities of amidinated fibrinogen acetylated to lesser extents, 1 and 0.25 molar excess *N*-acetylimidazole per mole of tyrosine was used and was reacted for 10 min at which time dialysis was begun at 0°.

Eighty-four per cent amidinated fibrin clots were formed by adding 2.5 units of thrombin to 4 ml of amidinated fibrinogen (8–10 mg/ml) in 0.02 M sodium barbital–0.15 M NaCl (pH 7.5). After 2 hr one of the clots was acetylated by dialysis against 50 ml of 0.02 M sodium barbital–0.15 M NaCl containing 0.375 g of *N*-acetylimidazole for 2 hr at 25°. The acetylated amidinated clot was then dialyzed against 0.02 M sodium barbital–0.15 M sodium chloride (pH 7.5) at 4° with three buffer changes. The acetylated amidinated clot was then dissolved in 3 M urea–0.15 M NaCl–0.02 M sodium barbital (pH 7.5) and its degree of acetylation was determined.

Acylation of soluble amidinated fibrin monomers in 2.5 M urea was performed by first adding 5 NIH units of thrombin solution to a solution of amidinated fibrinogen in 2.5 M urea and allowing thrombin to react 45 min at which time *N*-acetylimidazole was added as described for acetylation of amidinated fibrinogen. Excess reagent and side products were dialyzed out in the barbital buffer system made 2.5 M in urea.

In order to determine the actual amount of fibrin formed by action of thrombin in 2.5 M urea, a solution of amidinated fibrinogen and thrombin was prepared as for acetylation. At 45 min hirudin, a thrombin inhibitor (hirudin, Sigma), was added instead of *N*-acetylimidazole and the urea was removed by extensive dialysis against 0.15 M NaCl. The clot liquor was extruded and the per cent clotted was calculated relative to a control solution to which thrombin and hirudin were added separately but simultaneously.

The number of unmodified lysine residues was determined by trinitrophenylation by a method similar to Ozols and Strittmatter (1966). The difference between this value and that determined in the absence of acetylimidazole or ethyl acetimidate gave the number of lysine residues modified by these reagents. Percent clottability of fibrinogen and modified fibrinogen was determined as described by Laki (1951).

The number of acetylated tyrosines was determined by following the rate of deacetylation in 0.15–0.2 M NH_2OH–0.02 M sodium barbital (pH 7.5) by difference spectrophotometry at 278 nm similar to the method described by Simpson *et al.* (1963). The NH_2OH solution was made 50% in urea to solubilize acetylated amidinated fibrin for deacetylation.

The number of groups deacetylated by NH_2OH at pH 7.5 was also determined by measuring the number of moles of acetylhydroxamate formed by a procedure similar to that described by Balls and Wood (1956). To 1 ml of acetylated amidinated protein in 0.02 M sodium barbital–0.15 M NaCl (pH 7.5), 0.4 ml of 3.5 M NH_2OH (pH 7.0) was added. This solution was allowed to react 20 min. At this time 0.16 ml of 7 N HCl and 0.3 ml of 10% $FeCl_3$ in 0.1 N HCl were then added in sequence with agitation after each addition. The sample was filtered through a sintered glass filter and the optical density was read at 540 nm 10 min after the addition of ferric chloride. A standard curve was prepared using *p*-nitrophenyl acetate. Aliquots of *p*-nitrophenyl acetate in benzene were pipetted into test tubes. Benzene was then evaporated by a stream of dry nitrogen. Then 0.4 ml of NH_2OH (pH 7.0) and 1 ml of amidinated fibrinogen in 0.02 M sodium barbital–0.15 M NaCl (pH 7.5) were added. The standard was then treated as above.

Nitration of fibrinogen was performed similarly to the method described by Riordan *et al.* (1967). Tetranitromethane (Aldrich, 350 mol/mol of protein) was suspended in 8 ml of 0.01 M $NaPO_4$–0.15 M NaCl buffer (pH 8) mixed vigorously on a Vortex and then added to a stirring protein solution (1.0 mg/ml) in 0.05 M $NaPO_4$–0.15 M NaCl (pH 8) at 25°. Aliquots were removed at the indicated times, cysteine was added and the solutions were dialyzed against 0.01 M $NaPO_4$–0.15 M NaCl (pH 8) with three buffer changes. At 60 min a second addition of $C(NO_2)_4$ was made to the last aliquot and this solution was allowed to react 30 min longer. Nitration was also performed with a protein concentration of 0.5 mg/ml, with $C(NO_2)_4$ dissolved in ethanol and with termination of the reaction by partition on a Bio-Gel P-10 column. The nitrated protein fractions from the P-10 column were concentrated by ultrafiltration with an Amicon XM-300 membrane. The degree of nitration was determined by measuring the absorbance at 428 nm of the nitrated protein at pH 9. The absorbance at 600 nm was subtracted to correct for scatter. Nitrotyrosine was also determined on the long column of a Beckman 120 C amino acid analyzer (Sokolovsky *et al.*, 1966). Protein concentration was determined by microbiuret.

The p*K* of the nitrotyrosine groups in nitrated fibrinogen (1.6 groups/mol of fibrinogen) was determined by mixing aliquots of nitrated fibrinogen with 0.25 M sodium phosphate–0.25 M sodium borate buffer of various pH values. The final pH of the protein solutions was measured on an Instrumentation Laboratory pH meter. Changes of the nitrotyrosine adsorption at 428 nm as a function of pH were recorded on a Cary Model 14 spectrophotometer on the 0–0.1 slide-wire. The difference spectra were measured to correct for light scattering, using nitrated fibrinogen at pH 6.2 as the reference.

Clots were prepared for breaking weight measurements by adding 2.5 units of thrombin to 1 ml of modified fibrinogen (8 mg/ml) in 0.02 M sodium barbital–0.15 M NaCl buffer at pH 7.2 in a 1-cm test tube. The samples were allowed to clot 1 hr and 30 min and then treated as previously described (Phillips and York, 1973).

Samples of acetylated amidinated fibrinogen were deacetylated prior to clot formation in the following manner; 1-ml aliquots were dialyzed against 0.4 M NH_2OH in 0.02 M sodium barbital (pH 7.2) for 3 hr at 25°, the aliquots were then dialyzed against 0.02 M sodium barbital–0.15 M NaCl (pH 7.2) at 4° with four buffer changes. As a control, aliquots of amidinated fibrinogen were treated similarly.

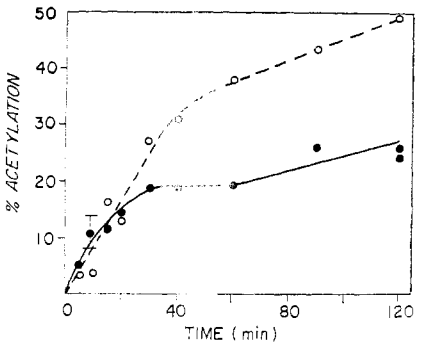

FIGURE 1: Rate of reaction of tyrosine and lysine in fibrinogen with *N*-acetylimidazole, 5-fold excess. Acylation was performed in 0.02 M sodium barbital–0.15 M NaCl (pH 7.5), 25° at a 5-fold excess reagent (30 mM) to tyrosine and lysine (1500 molar excess per fibrinogen): (○) per cent of the total lysine acetylated; (●) per cent of the total (100) tyrosine acetylated.

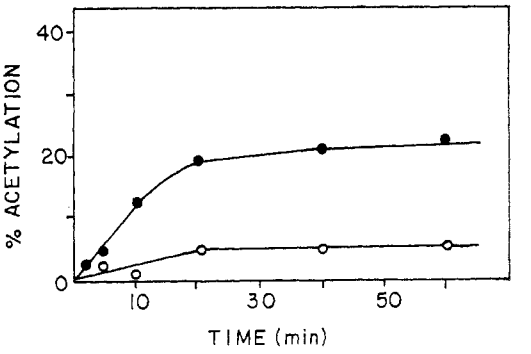

FIGURE 3: Rate of reaction of tyrosine and lysine in 84% amidinated fibrinogen with 5-fold molar excess of *N*-acetylimidazole (11 mM). Acylation conditions are as in the legend to Figure 1: (●) per cent tyrosine acetylated; (○) per cent lysine acetylated.

Results

The time course of the reaction of a fivefold molar excess of *N*-acetylimidazole per tyrosine and lysine (1500-fold excess per fibrinogen molecule) with bovine fibrinogen is shown in Figure 1 which illustrates that there is a group of 19 tyrosines which react within 30 min and a second group which begins to react at a slower rate at 60 min. In 40 min 30% of the lysines is acetylated at a fast rate and further reaction occurred at a slower rate. This slower rate of reaction of lysine after 40 min is probably due to the decrease in *N*-acetylimidazole concentration. The time course of the reaction of *N*-acetylimidazole (20-fold molar excess per tyrosine and lysine or a 6000-fold molar excess per fibrinogen molecule) with bovine fibrinogen is depicted in Figure 2. At this concentration of reagent the tyrosine curve is biphasic with 19 groups reacting quickly and 20 groups reacting at a slower rate. The break in the tyrosine curve is at 5 min at which time 25–30% (54–64 lysines) of the lysines has been acetylated. During the course of this reaction it was noted that the viscosity of the solution increased until a gel formed by 90 min. The data suggest that acetylation of lysine unfolds fibrinogen thus exposing more tyrosine groups. The rates of deacetylation of acetylated fibrinogen with hydroxylamine were all monophasic. A total of 87% of the lysine residues is acetylated. The rate of acetylation of lysine with the 6000-fold molar excess reagent per fibrinogen is pseudo-first-order up to about 60 min with a second-order rate constant of 0.43 M^{-1} min^{-1}. During the same time interval the kinetics of acetylation of tyrosine is biphasic (Figure 2). This result indicates that these exposed lysines are acetylated independently and are equally reactive to *N*-acetylimidazole. The unfolding during the time of the reaction due to acetylation of lysine was not sufficient to expose the remaining 10% of the lysines and 60% of the tyrosines. The number of lysines acetylated in fibrinogen by *N*-acetylimidazole at pH 7.5 agrees with the number of lysines modified by ethyl acetimidate at pH 8 to 8.5 (Phillips and York, 1973).

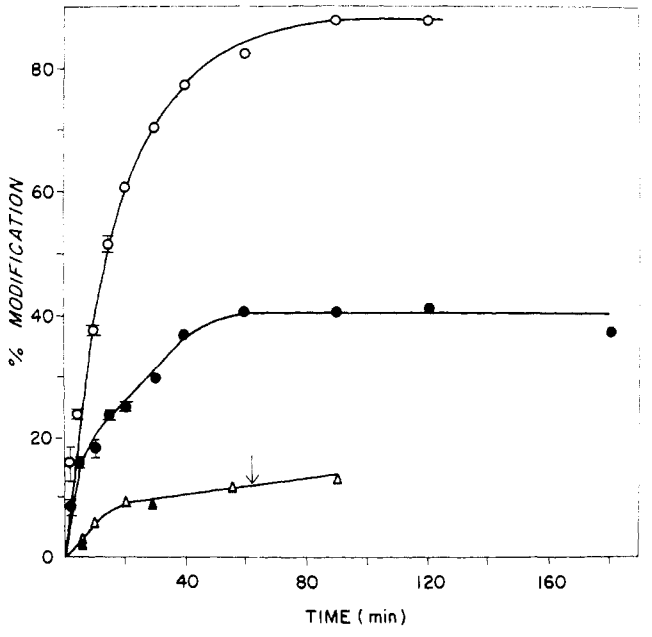

FIGURE 2: Rate of reaction of tyrosine and lysine in fibrinogen with *N*-acetylimidazole, 20-fold excess, and tetranitromethane. Acylation was performed in 0.02 M barbital–0.15 M NaCl (pH 7.5) 25° at a 20-fold molar excess reagent (120 mM) to tyrosine and lysine. The nitration was performed in 0.05 M $NaPO_4$–0.225 M NaCl (pH 8.0), 25° with a 350 molar excess reagent to protein: (○) per cent of the total lysine acetylated; (●) per cent of the tyrosine acetylated; (▲) per cent tyrosine nitrated in fibrinogen with a concentration of 0.5 mg/ml; (△) 1.0 mg/ml.

The time course for the reaction of tetranitromethane with bovine fibrinogen is shown in Figure 2. At 1 hr a second aliquot of $C(NO_2)_4$ was added; only two more tyrosine residues were nitrated in the next 30 min. Thus a total of 14.6 tyrosines is nitrated in 90 min. This curve shows that eight tyrosine residues are nitrated rapidly and six tyrosines at a slower rate. This value was confirmed by direct amino acid analysis of the 3-nitrotyrosine.

The time course of the reaction of *N*-acetylimidazole with 84% amidinated bovine fibrinogen is shown in Figure 3. The tyrosine curve shows that a maximum of only 22 groups reacted. The average number of tyrosines acetylated in six acetylation experiments of 84% amidinated fibrinogen was 20 ± 2. It is seen also from Figure 3 that there is a slow reaction with unamidinated lysine resulting in acetylation of 13 lysines (6%). This leaves ten per cent of the lysine residues unmodified even after amidination followed by acetylation. Ten per cent of the lysines remained unmodified after acetylation of fibrinogen (Figure 2).

If the reaction of 84% amidinated fibrinogen with fivefold molar excess *N*-acetylimidazole is carried out in 50% urea, 60 tyrosine residues are acetylated. Thus there was sufficient excess reagent for reaction if the tyrosine residues had been available in 84% amidinated fibrinogen in buffer.

TABLE I: Comparison of the Number of Tyrosine and Lysine Groups Acetylated at 1 hr as a Function of the Extent of Amidination of Fibrinogen.[a]

% Lys Amidinated	% Tyr Acetylated	% Lys Acetylated
0	19	38
85	22.5	6
90	22	

[a] Acetylation was at pH 7.5 with 5-fold molar excess per tyrosine and lysine for fibrinogen and 5-fold molar excess per tyrosine for amidinated fibrinogens.

If 90% amidinated fibrinogen is reacted with *N*-acetylimidazole, the same number of tyrosine residues are acetylated as for 85% amidinated fibrinogen and for fibrinogen as shown in Table I. Thus even 90% modification of lysine with ethyl acetimidate does not grossly unfold fibrinogen.

The effect of urea on the spectrophotometric assay for the number of tyrosines acetylated was determined and the result is shown in Table II. It is seen that the presence of urea does not affect the assay. Table II also shows that the number of tyrosines acetylated as determined by the spectrophotometric method agrees with the number found by the ferric hydroxamate assay within experimental error. This shows that only O-acetylated tyrosine is deacetylated under the deacetylation conditions used in these experiments.

The reaction of *N*-acetylimidazole (5-fold molar excess per tyrosine) with 84% amidinated fibrinogen as a function of urea concentration is shown in Figure 4. At less than 2 M urea 20% of the tyrosines is acetylated. As the urea concentration is increased it is seen that the number of tyrosine residues modified increases in a manner parallel to the change in conformation as determined by optical rotation (Mihalyi, 1965). At 3.5 M urea, 35% of the tyrosine residues is modified. A maximum of 89–100% of the tyrosine residues was acetylated in 8 M urea when a 20-fold molar excess of *N*-acetylimidazole was used. Thus essentially all the tyrosines are exposed by 8 M urea.

The reaction of *N*-acetylimidazole with amidinated bovine fibrin as a clot, and as soluble fibrin monomer prepared and acylated in 2.5 M urea is also shown in Figure 4. The number

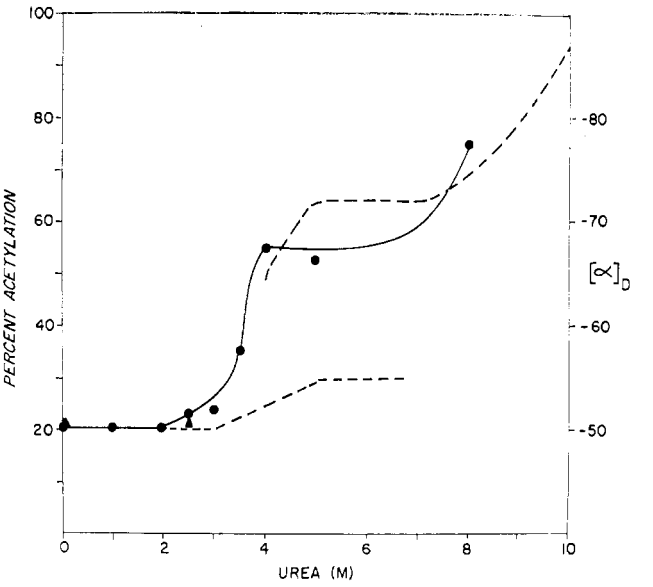

FIGURE 4: The effect of urea denaturation of amidinated fibrinogen on the number of tyrosines accessible to modification by *N*-acetylimidazole with 5-fold excess (11 mM) per tyrosine. Buffer conditions were as in Figure 1: (●) per cent accessible tyrosine at the urea concentration indicated; (- - - -) changes in the optical rotation of fibrinogen as a function of urea. The plot is from data of Mihalyi (1965) of urea effects on conformation of native and reversibly denatured fibrinogen, lower curve, and irreversibly denatured fibrinogen, upper curve. (▲) per cent accessible tyrosine in amidinated fibrin, no urea, and amidinated fibrin monomers, 2.5 M urea.

of tyrosine residues acetylated in each fibrin system agreed with the corresponding value found for fibrinogen. Seventy-five per cent of the amidinated fibrinogen was converted to amidinated fibrin monomer by thrombin in the polymerization inhibiting solvent (2.5 M urea).

We have found that reamidination of 84% amidinated fibrinogen of pH 8–8.3 resulted in a decrease of only 1–2% (2–4 lysines) in the groups available for trinitrophenylation in 8 M urea. However as shown in Figure 3, *N*-acetylimidazole reacts with an additional 13 lysine residues (6%) in 84% amidinated fibrinogen leaving approximately 21 (10%) of the lysines unacetylated or unamidinated and available for trinitrophenylation in 8 M urea.

Thus there is a large group of lysines of fibrinogen (90%) which can react with ethyl acetimidate-HCl at pH 8.5 (Phillips and York, 1973) and which react rapidly with *N*-acetylimidazole (Figure 2) and a second group (10%) which are readily exposed only in 8 M urea.

A normal p*K* of 7.25 (Riordan *et al.*, 1967) was determined for nitrated fibrinogen containing one to two nitrotyrosine residues.

Acetylation of 84% amidinated fibrinogen as described here does not change the clotting time or per cent clottability.

The breaking weights of acetylated amidinated fibrinogen as per cent of breaking weight of amidinated fibrinogen *vs.* acetylation of tyrosine is depicted in Figure 5a. These curves show that acetylation of amidinated fibrinogen causes the clots formed by addition of thrombin to be weaker than the control. Removal of the acetyl groups on tyrosine of acetylated amidinated fibrinogen with hydroxylamine at pH 7.5 causes a significant regeneration of the clot strength. The difference between the breaking weight of hydroxylamine-treated acetylated amidinated fibrinogen and untreated acetylated amidinated fibrinogen is the contribution of O-

TABLE II: Comparison of the Spectrophotometric Assay for O-Acetylated Tyrosine and Ferric Hydroxamate Assay and Effect of Urea on the Spectrophotometric Assay.

Protein	50% Urea	No. of Tyr Acylated/Mole of Fibrinogen: Spectrophotometric Method	No. of Tyr Acylated/Mole of Fibrinogen: Ferric Hydroxamate Method
85% Amidinated Sigma fibrinogen	–	19.4	22.8
Fraction I	+	19.4	
90% Amidinated Sigma fibrinogen	–	19.7	20.9 ± 0.3
Fraction I	+	19	

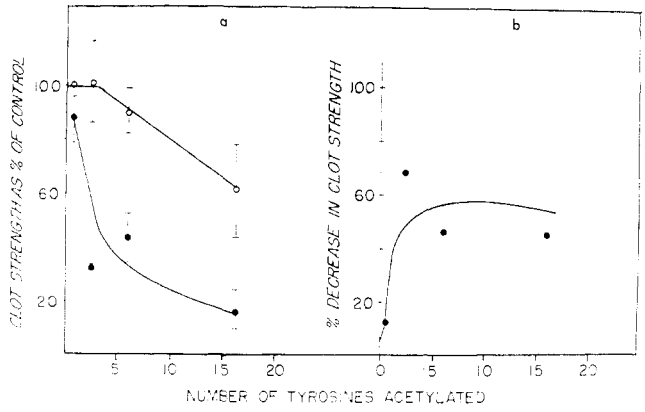

FIGURE 5: (a) Clot strength as per cent of the control *vs.* the number of tyrosines acetylated: (●) breaking weight of acetylated amidinated fibrinogen as per cent of control; (○) breaking weight of hydroxylamine-treated acetylated amidinated fibrinogen as per cent of control. (b) Per cent decrease in clot strength *vs.* number of tyrosines acetylated. The per cent decrease is found from the difference in the two curves in part a. The clots were 8 mg/ml in 0.02 M sodium barbital–0.15 M NaCl buffer (pH 7.2).

acetylation of tyrosine to the decrease in breaking weight. The plot of this difference is shown in Figure 5b. This curve demonstrates that the acetylation of only two tyrosine residues causes a 50% decrease in the breaking weight and that further acetylation of tyrosine has no effect on the breaking weight. However, as shown in Figure 3, there is some acetylation of lysine which probably accounts for the incomplete regeneration of clot strength observed in Figure 5a at higher degrees of acetylation of 84% amidinated fibrinogen.

Discussion

Concomitant with the acetylation of tyrosine is a much more extensive modification of the lysine residues at pH 7.5 with 35% (75 residues) of the lysine groups modified during the first phase of tyrosine acylation (Figure 2). Conversion of this many positively charged species to neutral species by acylation most probably results in a conformational change which exposes formerly buried tyrosine residues. The slower rate of acetylation of the second group of tyrosines may be a measure of the rate of unfolding of fibrinogen due to the change in charge on lysine upon acetylation. Therefore it appeared that all of those tyrosines in the slow phase of the acylation are unavailable in the native protein. Two approaches were utilized to determine if this thesis were unreasonable. First, an amidinated fibrinogen was prepared in which the lysines were not available for reaction with *N*-acetylimidazole and second, tetranitromethane was used to specifically modify accessible tyrosine.

Since there are no cysteine residues in fibrinogen, tetranitromethane becomes a reasonably specific probe for tyrosine. As can be seen (Figure 2) only 14.5 tyrosine residues reacted. This number agrees reasonably well with the 18–20 accessible tyrosine residues found in this study for the fast reaction of *N*-acetylimidazole with fibrinogen (the first rate for fibrinogen in Figure 1) and for the single rate observed for amidinated fibrinogen (Figure 3). Therefore, we conclude that in fibrinogen only 15–20 tyrosine residues are accessible rather than the 33–40 previously reported.

Fibrinogen is thought to exist as a dimer based on observations that it can be split into two identical sets of three different peptide chains (Clegg and Bailey, 1962; Henschen, 1964). If this model is correct there are only 7–10 tyrosine exposed per subunit. Evaluation of the effects of this modifi cation of a relatively small number of tyrosines, 7–10 out of a possible 100, on clotting time and properties of the clot be comes a meaningful exercise.

To demonstrate the availability of buried tyrosine residue in amidinated fibrinogen, acylation was performed in urea The number of tyrosines acetylated at 2 hr with fibrinoge (Figure 2) corresponds to that found in amidinated fibrinoge in 3.5 M urea. Only 0–10% of the tyrosines remained un modified in 8 M urea. The plot of *N*-acetylimidazole availabl tyrosine residues *vs.* urea concentration (Figure 4) is almos superimposable on the plot of conformation change *vs.* ure concentration (Mihalyi, 1965) suggesting that previous re ports of larger numbers of free tyrosine resulted from unfolc ing and exposure of buried tyrosines produced by acylatio of the lysine. In the pH titration studies (Huesby and Murray 1969) the number of exposed tyrosines may be high becaus of small changes of conformation which have been show to occur as the pH is increased (Mihalyi, 1965). Mihalyi (1965 showed that 3.5 M urea causes a small perturbation of th structure which is reversible upon removal of the urea Therefore, the increase in tyrosine residues acetylated in 3. M urea is a result of a very small change in conformation.

This increased accessibility of tyrosine by mild perturbatio in 3.5 M urea, suggests that these newly accessible tyrosine may be on the surface of the molecule but hindered fror reacting by hydrogen bonding or that these tyrosines may b partially buried on the surface. These data suggest that th structure of fibrinogen is very fragile and that physiochemica studies which cause a change in charge either by titration c chemical modification may be very unreliable.

The data suggest that even small changes in optical rotator dispersion (ORD) represents a large exposure of tyrosine and that the number of available tyrosines can serve as sensitive indicator for conformational changes in fibrinoger Thus if a conformational change occurs in the transformatio of fibrinogen to fibrin, one would expect to see a change in th number of tyrosine residues exposed. However, the dat presented here show that amidinated fibrin monomer in 2.5 urea or amidinated fibrin clot have the same number of ex posed tyrosines as for amidinated fibrinogen. This indicate that no conformational change occurs on conversion c fibrinogen to fibrin which is in agreement with the results c optical rotation found by Kay and Marsh (1961) on the tran formation of fibrinogen to fibrin. The data presented here als suggest that if tyrosine residues are involved in polymerizatio they are equally accessible in fibrinogen as well as fibrin.

The normal p*K* found for the nitrotyrosine in fibrinoge containing one to two nitrotyrosine residues is good evidenc that the exposed unmodified tyrosines have a normal p*K* c 9.5–10. This is interesting in view of the report of Mihalyi an Albert (1971) that one to two tyrosines in fibrinogen can b dansylated even at pH 6.8 with dansyl bound to Celite. Th may indicate that some exposed tyrosines are in a partial hydrophobic environment which may bind dansyl and thu increase the local concentration of the reagent, thereby en hancing the rate of dansylation.

It is significant that out of the 100 possible tyrosine residue acetylation of only two to three tyrosines (Figure 5b) causes 50% decrease in the breaking weight of the acetylated amid nated fibrin clot. Since O-acetylation of tyrosine preven hydrogen bonding through the phenolic hydroxyl, these da suggest a role for tyrosine in maintaining clot strength b hydrogen bonding. However, hydrogen bonding by the

tyrosines is not the driving force for fibrin monomer aggregation since clottability and clotting time are not changed by acetylation of amidinated fibrinogen. It seems plausable to suggest that the driving force for aggregation may be electrostatic interactions which come into play when the fibrinopeptides are removed and that hydrogen bonding of tyrosine acts to align the monomers in the most stable arrangement. A good analog might be the base paring of nucleotides. Similarly Ferry *et al.* (1954) have suggested that charge patterns are probably largely responsible for orientating the monomer units for polymerization and that hydrogen bonds and other short-range attractions may also contribute. A mechanism for polymerization encompassing both hydrogen-bonding and electrostatic interactions is attractive based on the data presented here and also on the work of Shulman and Ferry (1950), which showed a large effect of ionic strength on the opacity–pH transition of fibrin. Furthermore, recent attempts (Endres and Scheraga, 1966) to explain the thermodynamics of clot formation based solely on hydrogen bonding have not been successful. Collen *et al.* (1970) found that the polymerization of fibrin monomers is accompanied by a volume increase which could not be explained by hydrogen bonding alone and suggests hydrophobic bonding or charge neutralization. Collen *et al.* (1970) found no binding of 8-anilino-1-naphthalenesulfonate which suggests the lack of a surface hydrophobic site.

The data presented here show a possible role for the phenolic hydroxyl of tyrosine in strengthening the fibrin clot *via* hydrogen bonding and that this is not the driving force for aggregation of fibrin monomers.

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