The Quantitation of Isotope Loss with Tyrosine Loss in the Canine Fibrinogen–Fibrin Transformation (35659)

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The addition of thrombin to canine fibrinogen results in the loss of several peptides which contain tyrosine residues (1–3). In the labeling of fibrinogen with iodine (^{131}I), these residues are iodinated. An exact knowledge of this loss of iodotyrosine-containing peptides during the canine fibrinogen–fibrin transformation is essential for interpreting the results of fibrinogen-turnover studies and for the quantitation of fibrinogen concentration by an isotope dilution technique (4, 5). Regoeczi and Walton (6) have reported that approximately 11% of the ^{125}I label incorporated into canine fibrinogen is lost during coagulation due to thrombin addition, whereas, Zetterquist (7) has reported a loss of 16%.

This communication reports the results of experiments to determine both the radioactivity and the amount of tyrosine in highly purified ^{131}I-labeled canine fibrinogen as well as in the fibrin collected from this fibrinogen after thrombin addition. From the differences between these results on fibrinogen and fibrin, both the losses of radioactivity and tyrosine can be calculated. These experiments were undertaken, therefore, not only to quantitatively determine the loss of radioactivity during the fibrinogen–fibrin transformation but also to establish the relationship between this isotope loss and the simultaneous loss of tyrosine.

Methods. A. Fibrinogen preparation. The fibrinogen used in this study was isolated from the plasma of mongrel dogs by the method of Atencio *et al.* (8). The purified fibrinogen was dialyzed against four 1-liter portions of 0.005 *M* sodium citrate over a 40-hr period at 4° to remove both the ammonium sulfate and any cold insoluble material. After dialysis, the fibrinogen solution was clarified by centrifugation and the concentration determined by the measurement of the optical density at 280 mμ and the use of the extinction coefficient ($E^{0.1\%}_{1cm} = 1.583$) determined for the dog (9).

B. ^{131}I-labeling procedure. A volume of the fibrinogen solution containing approximately 10 mg was iodinated by the iodine monochloride method (8, 10) at a mean substitution level of 0.5–1.0 atoms ^{131}I/fibrinogen molecule. The free ^{131}I was reduced to less than 1% of the activity in association with the protein by repeated dialysis against 0.005 *M* sodium citrate.

C. Coagulability measurements. 1. *Ultraviolet absorption at 280 mμ.* The coagulability of the purified and labeled canine fibrinogen was determined by measurement of the UV absorption at 280 mμ as described by Atencio *et al.* (8).

2. *Radioactive measurements.* Coagulability can also be measured as the percentage of the radioactivity of the labeled fibrinogen preparation which remains in association with fibrin after thrombin addition. This recovery of the radioactivity in the clot was determined by following each of two procedures used in this laboratory for the quantitative determination of fibrinogen: (a) an isotope-dilution method (8); and (b) a colorimetric method (11).

a. Clotting procedure for isotope-dilution method. To 4.5 ml of 0.005 *M* sodium citrate was added 0.5 ml of the labeled fibrinogen, and the fibrinogen was precipitated by the

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addition of 1.7 ml of 4 *M* $(NH_4)_2SO_4$. The precipitate was washed once with 1 *M* $(NH_4)_2SO_4$ and then dissolved in 4 ml of 0.005 *M* sodium citrate. An 1.0-ml portion of this solution was counted for ^{131}I activity (r_1, cpm) in a well-type scintillation counter (Packard Instrument Co., Downers Grove, Ill.). After the counting of r_1, 2 ml of 0.005 *M* sodium citrate and 0.2 ml of a thrombin (Parke Davis Co., Inc., Detroit, Mich.) solution (containing 4 NIH units) were added to the 1.0 ml of fibrinogen solution in the counting tube. The fibrin was carefully collected on a glass rod. After the removal of the fibrin-containing glass rod, the remaining liquid in the counting tube was centrifuged and 1 ml was counted for ^{131}I activity. The total activity in the clot supernatant (r_2, cpm) was determined by correcting for the dilution. The fibrin on the glass rod was rinsed with distilled water with care being taken not to dislodge the fibrin mass. The fibrin was then dissolved into 4 ml of a freshly prepared alkaline urea solution (40% urea dissolved in 0.2 *N* NaOH) and the ^{131}I activity was determined in a 1.0 ml sample. The total activity in association with the fibrin (r_3, cpm) can thus be calculated. The percentage of radioactivity recovered in the fibrin clot after the clotting procedure used in an isotope-dilution method is calculated as:

$$C = \left(\frac{r_3}{r_1} \right) 100. \qquad (1)$$

b. Clotting procedure for colorimetric method. To graduated 40-ml glass centrifuge tubes was added 0.5 ml of finely pulverized glass (Scientific Glass Apparatus Co., Bloomfield, N. J., Pyrex 20–80 mesh), 10 ml of 0.9% NaCl, 0.5 ml of the labeled fibrinogen preparation (containing a total radioactivity of r_1 cpm) and 0.1 ml of a thrombin solution containing a total of 15 NIH units. The 40-ml centrifuge tube was gently swirled until the fibrin was intertwined in the ground glass. Several minutes later, the tube was swirled again for further collection of the fibrin. This procedure was repeated several times until there appeared to be no further collection of the fibrin. After centrifugation to compress the clot into the ground glass, 1 ml of the clot supernatant was counted for ^{131}I activity and the total activity in the supernatant (r_2) was calculated by correcting for the dilution. The remaining supernatant fluid was then discarded and the fibrin collected on the ground glass was washed five times with 15-ml volumes of 0.9% NaCl, with care being taken not to lose any of the fibrin. After the addition of 1 ml of a 10% NaOH solution, the tube was placed into a boiling water bath for exactly 10 min. After the removal of the tube from the water bath, 10 ml of distilled water was added to the tube and mixed with the hydrolyzed fibrin. The tube was allowed to come to room temperature and the volume in the tube was brought up to 25 ml (including the 0.5 ml of pulverized glass) with distilled water and the contents were mixed thoroughly. One ml of this solution was counted for radioactivity and the total activity in association with the fibrin (r_3) was calculated by correction for the dilution (24.5 ml). The total activity contained in r_3, when expressed as the percentage of the activity contained in the original 0.5-ml sample (r_1) gives the recovery of radioactivity in the fibrin clot after the clotting procedure used in a colorimetric method [see Eq. (1) above].

3. *Tyrosine determinations.* Tyrosine in the purified fibrinogen and fibrin preparation was determined by a colorimetric method routinely used for the quantitation of fibrinogen (11) which makes use of the procedure described by Folin and Ciocalteu (12) for the measurement of tyrosine in proteins. The amount of tyrosine in a protein is determined by reference to a standard curve constructed from known amounts of purified tyrosine.

a. *Tyrosine in purified fibrinogen and fibrin.* Aliquots containing identical amounts of purified fibrinogen were pipetted into two 40-ml graduated glass centrifuge tubes containing 0.5 ml of pulverized glass and 10 ml of 0.9% NaCl. Thrombin was added to one of the centrifuge tubes and the fibrin was collected and washed as described above (C, 2b). To each tube, one containing the purified fibrinogen and the other containing the washed fibrin from an identical aliquot, was added 1.0 ml of a 10% NaOH solution

and both were placed into a boiling water bath for 10 min to hydrolyze the protein. After the hydrolysis, addition of distilled water, and adjustment to room temperature, the content of each tube was quantitatively transferred into a 25-ml volumetric flask. Care was taken to assure that the volume in the flask did not exceed approximately 20 ml. To the flask was then added 3.0 ml of 20% Na_2CO_3 solution, followed by 1.0 ml of 2 *N* phenol reagent solution (Fisher Scientific Co., Springfield, N. J.) and the time of the phenol reagent addition was carefully noted. The content of the flask was mixed carefully after each addition and the volume was finally adjusted to 25 ml with distilled water. The color of the solution was allowed to develop for exactly 30 min, at which time the optical density was measured at 650 mμ on a Model B spectrophotometer (Beckman Instrument Co., Fullerton, Calif.) against a blank containing no fibrinogen but carried through the entire procedure outlined above.

b. *Standard tyrosine curve.* Two hundred mg of purified tyrosine (Sigma Chemical Co., St. Louis, Mo.) were dissolved in 1 liter of 0.1 *N* HCl. Aliquots containing 0.02–0.18 mg of this amino acid were pipetted into 25-ml volumetric flasks. To each flask was added 1 ml of 10% NaOH, 3.0 ml of 20% Na_2CO_3, 1 ml of 2 *N* phenol reagent, followed by distilled water to bring the volume to 25.0 ml. All optical densities were measured at exactly 30 min after the addition of phenol reagent, and the readings are related to the quantities of tyrosine (mg) added to the 25-ml flasks.

D. Nitrogen quantitation. Fibrinogen was quantitated by determination of total nitrogen (13). The fibrinogen-containing solutions, blanks, and standard solutions containing known amounts of protein nitrogen (Protein Standard Solution, Lot No. C4508, Armour Pharmaceutical Co., Kankakee, Ill., distributed by Fisher Scientific Co., Springfield, N.J.) were pipetted into Kjeldahl flasks containing approximately 500 mg of a mixture of K_2SO_4 and mercuric sulfate (as a catalyst) and 1 ml of concentrate H_2SO_4, and the flask contents were digested for 3 hr. The distillation was performed with a thiosulfate-containing NaOH solution, and approximately 50 mg of powdered zinc were added to each flask to free any nitrogen which might be present as a complex salt of mercury. The distillate was collected in a boric acid solution containing a mixture of bromocresol green and vital red as indicator. Titration was performed with 0.01 *N* HCl, and the samples containing known amounts of protein nitrogen were used to standardize the end point color and thus to make any correction on the titration volume of the fibrinogen samples, which might result from inconsistencies in end point judgment from one series of experiments to another. A factor of 5.92 is used to convert the nitrogen content to fibrinogen concentration. This factor is obtained from the percentage of nitrogen (16.9%) in human (14), bovine (15), and canine fibrinogen (to be published).

Results. A. Coagulability by ultraviolet measurement. The coagulability of canine fibrinogen by UV measurement at 280 mμ on 10 samples has an average value of 95.1 ± 1.5%. The individual and average values as well as standard deviations are listed in Table I.

B. Coagulability by radioactivity measurement. a. Clotting procedure for isotope-dilution method. The individual and average values for the percentage of the radioactivity present on the fibrinogen molecule which can

TABLE I. Coagulability By UV Measurement.[a]

Sample	d_1	(d_2-d_3)	C_{UV}
1	0.315	0.013	95.9
2	0.315	0.016	94.9
3	0.315	0.013	95.9
4	0.315	0.013	95.9
5	0.315	0.024	92.4
6	0.315	0.015	95.2
7	0.315	0.013	95.9
8	0.315	0.024	92.4
9	0.315	0.014	95.6
10	0.315	0.009	97.1
Mean		0.015	95.1
SD			1.5

[a] d_1, optical density of original fibrinogen solution; d_2, optical density of the supernatant fluid after fibrin removal; d_3, optical density of the thrombin added to a blank; C_{UV} $100-100(d_2-d_3)/d_1$.

TABLE II. Coagulability By Radioactivity Measurement.

Sample	Isotope-dilution procedure			Colorimetric procedure		
	% in Fibrin r_3/r_1	% in supernatant r_2/r_1	Total % $(r_2+r_3)/r_1$	% in Fibrin r_3/r_1	% in supernatant r_2/r_1	Total % $(r_2+r_3)/r_1$
1	83.6	15.4	99.0	79.3	18.5	97.8
2	85.2	15.3	100.5	84.1	18.0	102.1
3	83.8	15.7	99.5	82.7	17.3	100.0
4	86.0	15.5	101.5	84.5	17.2	101.7
5	84.0	15.7	99.7	85.1	17.1	102.2
6	83.1	15.8	98.9	86.6	16.7	103.3
7	84.9	15.4	100.3	87.5	17.1	104.6
8	85.5	15.4	100.9	86.0	17.0	103.0
9	84.6	15.1	99.7	85.6	16.5	102.1
10	89.6	15.4	105.0	89.6	17.5	107.1
Mean	85.0	15.5	100.5	85.1	17.3	102.4
± SD	1.8	0.2	1.8	2.8	0.6	2.5

be recovered in fibrin as well as in the supernatant fluid resulting from clot removal are listed in Table II. The percentage of the fibrinogen radioactivity recoverable in fibrin is 85.0 ± 1.8%, and in the supernatant fluid is 15.5 ± 0.2%. The total radioisotope recovered thus averages 100.5 ± 1.8%.

b. Clotting procedure for colorimetric method. The individual and average average values for the percentage of the fibrinogen radioactivity present in fibrin and in the supernatant fluid obtained in a colorimetric method are also listed in Table II. The fibrin contains 85.1 ± 2.8% of the activity present in fibrinogen and 17.3 ± 0.6% remains in the supernatant fluid, with a total of 102.4 ± 2.5% of the initial activity being recoverable.

C. Tyrosine determinations. 1. Standard curve. The optical density at 650 mμ of solutions containing 0.02–0.18 mg of purified tyrosine/25.0 ml are plotted in Fig. 1. Least-square analysis of the data points reveals that the equation $y = 2.557x$ is the line of best fit with a correlation coefficient of

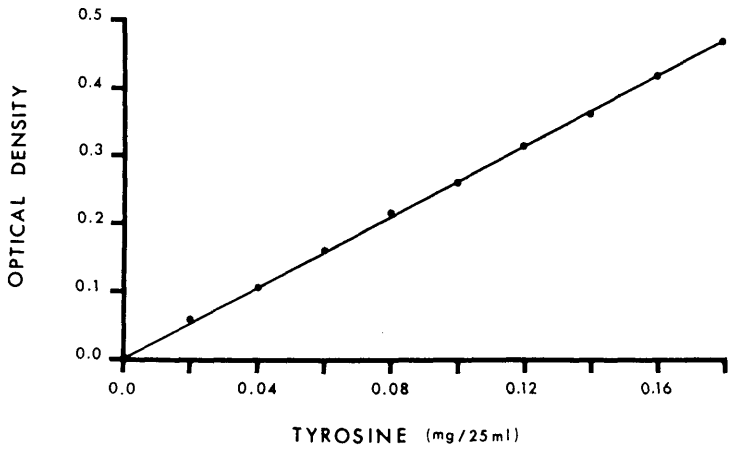

FIG. 1. The optical density at 650 mμ of purified tyrosine in 25-ml fluid volume. Each point represents the average of six determinations. The least-square analysis equation of best fit is $y = 2.557x$ with a correlation coefficient of 0.9997. All densities were determined exactly 30 min after color development.

0.9997. The milligrams of tyrosine in (a) a fibrinogen sample; or (b) in the fibrin collected after thrombin addition to an identical fibrinogen sample, both contained in a 25-ml volume, can thus be obtained from the measurement of the optical density at 650 mμ and the use of this value of 2.557.

The time for color development (30 min) was established by scanning these tyrosine and hydrolyzed fibrinogen solutions from 550 to 800 mμ (Beckman ratio recording spectrophotometer model DK II) at different times after the addition of the phenol reagent. Figure 2 shows the results of one such experiment, in which the optical density of both solutions increased to a maximum value at approximately 10–15 min after the addition of the phenol reagent and remained reasonably stable up to approximately 40 min. The 30-min time allowed for color development is thus sufficient to reach a stable value for the purified tyrosine, and tyrosine liberated from fibrinogen by alkaline hydrolysis. As shown the peak of absorption (Fig. 2) occurred at approximately 740 mμ in each case instead of 650 mμ, the wavelength commonly used for

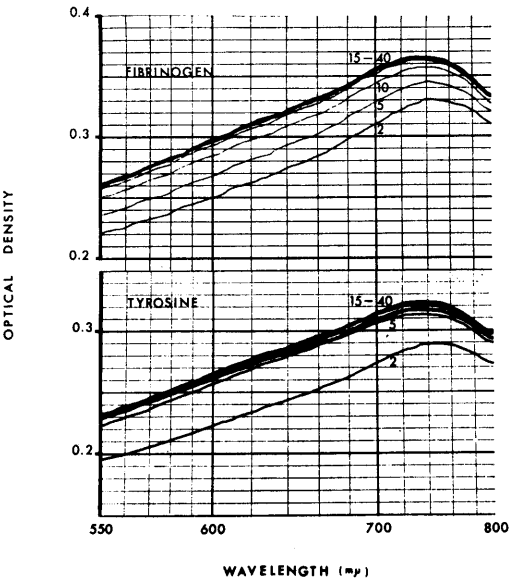

FIG. 2. The optical densities from 550 to 800 mμ of a purified canine fibrinogen (1.5 mg/25 ml) and purified tyrosine (0.11 mg/25 ml) at 2, 5, 10, 15–20–30–40 min after color development. Note that the curves rise to a maximum value at approximately 15 min after phenol addition and also that the maximum peak of absorption occurs at approximately 740 mμ.

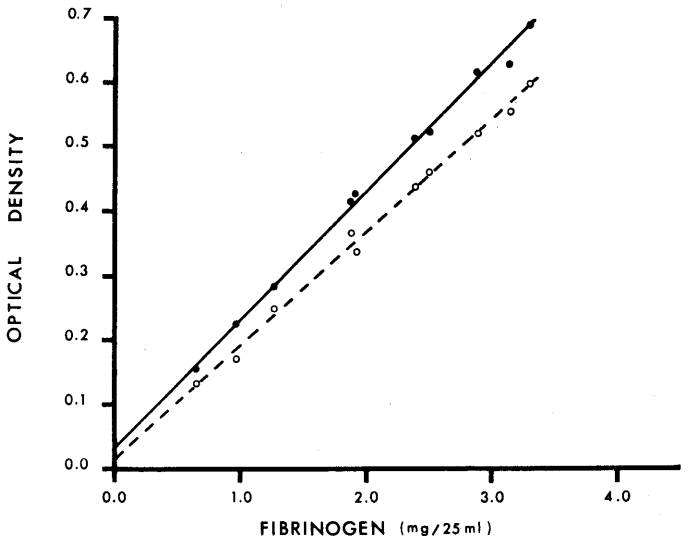

FIG. 3. The optical density of purified canine fibrinogen dissolved in 25 ml (●); and of the fibrin, collected from an identical fibrinogen-containing solution after the addition of thrombin, also contained in 25 ml (○) plotted against the measured amount of fibrinogen. (—, - - -) represent the least-square analysis best fit to the experimentally determined points. The correlation coefficient between OD and fibrinogen concentration for the fibrinogen-containing samples is 0.998 and for the fibrin-containing samples is 0.997.

tyrosine determination in the quantitation of fibrinogen (11).

2. *Tyrosine determination on fibrinogen- and fibrin-containing solutions.* The optical density of varying amounts of purified canine fibrinogen dissolved in 25 ml and the optical density of the fibrin collected from the same amounts of fibrinogen and dissolved in 25 ml are both plotted in Fig. 3 as a function of the amount of fibrinogen calculated from nitrogen determination. The equation describing the best fit to the fibrinogen data calculated from least-square analysis is $y = 0.1961x + 0.036$ with a correlation coefficient of 0.998, and for the fibrin data is $y = 0.1736x + 0.017$ with a correlation coefficient of 0.997. The curves are linear over the range of fibrinogen studied and do not pass through the origin.

If each value of optical density in Fig. 3 is corrected for the intercepts and then divided by 2.557, the amount of tyrosine present in the fibrinogen as well as the resulting fibrin is obtained (Table III). The percentage of the tyrosine which is retained in fibrin is 89.4 ± 4.8%. The percentage of the tyrosine which is lost during the fibrinogen–fibrin transformation thus averages 10.6 (Table III).

Discussion. The fibrinogen preparations used in this study were high in purity as can be seen from the UV coagulability at 280 mμ. This average value of 95.1% of thrombin-coagulable protein observed in this study has been routinely observed for canine fibrinogen and agrees well with the coagulability determined by other methods on similarly purified dog fibrinogen preparations (16).

Utilizing this purified canine fibrinogen and the two clotting procedures described it has been shown that, on the average, 16% of the radioiodine in association with the fibrinogen molecule is set free into the super-

TABLE III. Coagulability By Tyrosine Measurements.

Sample	Fibrinogen (mg)	Tyrosine (mg)		Tyrosine % retained in fibrin	Tyrosine % lost
		Fibrinogen	Fibrin		
1	0.650	0.046	0.045	97.8	2.2
		0.047	0.045	95.7	4.3
2	0.975	0.073	0.060	82.2	17.8
		0.074	0.063	85.1	14.9
3	1.270	0.097	0.092	94.9	5.1
		0.096	0.090	93.8	6.2
4	1.890	0.150	0.141	94.0	6.0
		0.147	0.136	92.5	7.5
5	1.925	0.158	0.127	80.4	19.6
		0.149	0.123	82.6	17.4
6	2.400	0.189	0.163	86.2	13.8
		0.185	0.166	89.7	10.3
7	2.510	0.191	0.169	88.5	11.5
		0.192	0.177	92.2	7.8
8	2.900	0.228	0.197	86.4	13.6
		0.226	0.196	86.7	13.3
9	3.160	0.226	0.205	90.7	9.3
		0.238	0.214	89.9	10.1
10	3.320	0.252	0.227	90.1	9.9
		0.259	0.228	88.0	12.0
Mean				89.4	10.6
± SD				4.8	4.8
Range				80.4–97.8	2.2–19.6

natant fluid during the fibrinogen–fibrin transformation after thrombin addition. The average value found in this study agrees closely with the value of 16% reported by Zetterquist (7). It would thus appear that approximately 84% of the total radioisotope present in labeled canine fibrinogen is retained in the fibrin resulting from thrombin addition, at least at the mean substitution levels used in these studies: 0.5–1.0 atom of ^{131}I/molecule of fibrinogen in the present study, and 0.8–2.7 atom/molecule in the study of Zetterquist (7).

The finding that approximately 10.6% of the tyrosine of canine fibrinogen is present in the peptides split off after thrombin addition suggests that at least this percentage of isotope loss can be accounted for as iodotyrosine. This value agrees closely with the value of 11.1% isotope loss reported for canine fibrinogen by Regoeczi and Walton (6). These authors postulated that this isotope loss could be accounted for by the presence of 2 nonsulfated tyrosine residues present in the B peptide. This was based on the observation that sheep and goat fibrinogen, which are known to contain 1 free and 1 sulfated or esterified tyrosine, shows a 5.6% loss of isotope after thrombin addition. This postulate was confirmed by the work of Osbahr *et al.* (3) who found that 3 peptides are released from canine fibrinogen, one of which (peptide III) is exactly similar in amino acid sequence to peptide B of other workers (1), but contained 2 nonesterified tyrosine residues. Zetterquist (7) reported that 9% of the isotope loss can be recovered in association with the split-off peptides and this also agrees with the 10.6% tyrosine loss observed in this study. The work of Zetterquist (7) defines the B peptide as being made up of two components: B_1 containing 1 free and 1 sulfated tyrosine, and B_2 which contains 2 sulfated tyrosine residues. These data conflict with that of Osbahr *et al.* (3) and places in question the actual state of the tyrosines which are split off from canine fibrinogen. The state of the tyrosine residues in an important point which should be firmly established since both Zetterquist (7) and Regoeczi and Walton (6) have found that sulfated tyrosines are not labeled during the iodination procedure.

The remaining 4–5% of the total isotope which is lost from canine fibrinogen after thrombin addition, but which is unaccounted for by quantitation with tyrosine loss may be in association with the histidine residue present in peptide B. The presence of monoiodohistidine has been demonstrated by thin-layer chromatography of an alkaline hydrolysate of fibrinopeptide B_1 (7). A possible explanation of why Regoeczci and Walton (6) did not report a total of 16% may be because the mean substitution level of iodine was the lowest (<0.5 atoms/fibrinogen molecule) of the three studies and the histidines may thus not have been labeled. The quantitation of any iodine loss in association with histidine is in need of further investigation.

Summary. Fibrinogen from the plasma of mongrel dogs was isolated and purified by fractional precipitation with ammonium sulfate. Cold-insoluble material and ammonium sulfate were removed by dialysis at 4° against 0.005 *M* sodium citrate. Portions of the resulting highly purified fibrinogen (95.1% clottability by 280 mμ analysis) were labeled with ^{131}I by the iodine monochloride method. The percentage of the protein-bound radioactivity which (i) remained in association with the collected fibrin after thrombin addition; and (ii) was released in association with the split-off peptides into the clot supernatant fluid was determined by utilizing two different clotting procedures. The clotting procedure commonly used in an isotope dilution technique for fibrinogen quantitation has shown that 85% of the radioactivity remains associated with fibrin and the remaining 15.5% is recoverable in the supernatant fluid. The utilization of a clotting procedure for the colorimetric determination of fibrinogen in which the color is a measure of the amino acid tyrosine has revealed that 85.1% of the radioactivity can be recovered in fibrin with 17.3% being released into the supernatant fluid. These results suggest that approximately 16% of the radioactivity of labeled canine fibrinogen is released in the fibrinogen–fibrin transformation. The simultaneous loss of tyrosine from the fibrinogen molecule in the

formation of fibrin has been found to be 10.6% and thus of the 16% total isotope loss at least this amount can be accounted for as iodotyrosine. The remaining 4–5% is at present still unaccounted for, but may be in association with the histidine residues present in the split-off peptides.

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