Formation of Complexes between Protein and Tannic Acid

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Tannic acid and protein interact to form soluble and insoluble complexes, and the latter are favored by a pH near the isoelectric point of the protein and an excess of tannic acid. As the concentrations decrease, higher ratios of tannin to protein are possible in soluble complexes. The number of sites on a gelatin molecule occupied per tannin molecule decreases as the tannin concentration increases, and this is accompanied by a decrease in the apparent equilibrium constant of binding between the tannic acid and the gelatin. Bonding between gelatin and tannin molecules is strong, particularly when a greater weight of gelatin than tannin is present; hence, there is relatively little unbound tannin in such soluble systems. Tannic acid is heterogeneous with regard to its ability to precipitate gelatin. Under no conditions could all the added tannic acid be precipitated by gelatin.

An understanding of the interactions between tannins and proteins is of importance for effective control and removal of haze in natural beverages. Much of our knowledge of these reactions comes from work in the field of leather research (Gustavson, 1956); however, the conditions of the studies often involved a large excess of tannin with no protein remaining in solution, while protein-tannin hazes in beverages involve soluble as well as insoluble protein-tannin complexes.

The types of protein and tannin affect the reaction. Greater precipitation occurs with high molecular weight gelatin (Hrazdina *et al.*, 1969). Tannic acid contains a mixture of phenolics (White, 1958), and Page (1942) has suggested that those of larger molecular weight combined more strongly with gelatin. Despite this heterogeneity, it has become clear that the binding between tannins and proteins was largely by hydrogen bond formation between the phenolic hydroxyl groups of the tannins and the carbonyl groups of the protein peptide bonds (Cannon, 1955; Grassmann, 1937; Gustavson, 1954).

Work reported earlier (Calderon *et al.*, 1968) has shown that the interaction between tannin and protein resulted in the formation of both soluble and insoluble complexes, and the distribution of reactants was influenced by alcohol, salt, and pH, as well as the type of tannin. In the present report the effects of absolute and relative concentrations of tannic acid and gelatin on the composition of the soluble and insoluble reaction products are presented. Apparently an extremely mixed population of complexes was present with regard to both the number of tannic acid molecules combined with one gelatin molecule and the number of gelatin molecules present in a complex. The composition of the soluble portion was as important as the insoluble portion, since it represented conditions and concentrations where tannic acid and proteins were in equilibrium in one phase. The composition of the soluble phase suggested that the equilibrium was strongly dependent upon concentration, while conditions of complete precipitation showed that there were neighboring group interactions. These ideas were tested under conditions where reactant heterogeneity and the uncertainties due to the soluble and insoluble complexes were brought into consideration.

PROCEDURE

Tannic acid (Mallinckrodt analytical reagent) was purified by fractional precipitation in ethyl acetate–hexane to remove extraordinarily large and small components. The material collected was soluble in 20 parts of ethyl acetate to 3 parts of hexane (v./v.) and insoluble in 20 parts of ethyl acetate to 10 parts of hexane. Its average osmotically determined molecular weight was 1100. Chromatography with 5% 1-butanol on cellulose thin layers showed about 5% remaining at the start point and about 5% with an R_f greater than 0.40.

Calfskin gelatin (Eastman Kodak) was a high molecular weight product with negligible ash, having an isoelectric point of 5.2. At a concentration of 2 grams per 100 ml. at pH 7.0, it had a specific viscosity of 1.6. The molecular size in this preparation was homogeneous as determined by gel filtration (Hrazdina *et al.*, 1968).

Tannic acid was routinely measured using the Folin-Ciocalteu reagent (Singleton and Rossi, 1965). Protein was determined by the turbidometric procedure of Mejbaum-Katzenellenbogen (1955). Throughout this work, pH was adjusted through the use of HCl or NaOH.

In carrying out the experiments the tannic acid was always added to a well-stirred gelatin solution. This order of addition avoided the premature formation of coarse precipitates. The supernatants of the tannic acid–gelatin mixtures were the liquid phases present after centrifugation at 90,000 × G for 30 minutes. Polyolefin centrifuge tubes were preferable to tannin-absorbing cellulose nitrate tubes. All analyses were carried out on the supernatant solutions, and the precipitate compositions were obtained through calculation.

Polyvinylpyrrolidone (Polyclar AT powder, General Aniline and Film Corp.) was freed of its very fine materials by a five-fold repeated sequence of suspensions in 20 times its weight of water, partial settling, and decantation. The final residue was frozen and lyophilized.

A modification of the notational convention of Klotz (1953) was used to describe the protein–tannic acid complexes:

R = grams of precipitated tannic acid per gram of precipitated protein

R' = grams of supernatant tannic acid per gram of supernatant protein

Dinitrophenylation of Gelatin. Two grams of calfskin gelatin were dissolved in 200 ml. of water containing 5 grams of finely ground barium carbonate. One milliliter of 1-fluoro-2,4-dinitrobenzene was added dropwise over a 2-hour period with continuous stirring; then the mixture was neutralized with dilute H_2SO_4, and centrifuged. The supernatant was extracted with ethyl ether to remove unreacted fluorodinitrobenzene. The aqueous phase was evaporated in a vacuum rotary evaporator, redissolved in water, then lyophilized to give a fluffy yellow product. A solution of the DNP-gelatin containing 1 mg. per ml., pH 3.0, had an

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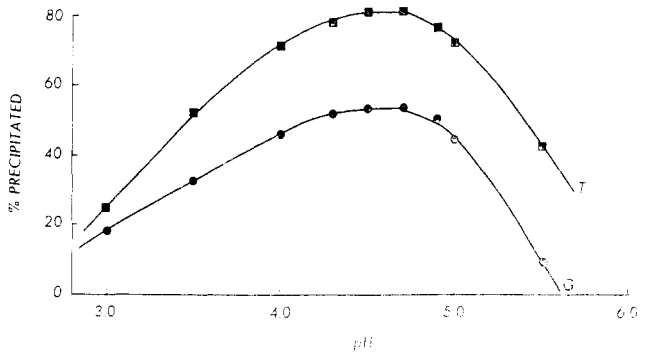

Figure 1. Effect of pH on per cent precipitation of gelatin and tannic acid

Initial concentrations, 100 mg. gelatin/100 ml., 25 mg. tannic acid/100 ml. ■ % tannic acid precipitated ● % gelatin precipitated

absorbance of 0.65 at 435 mμ. The isoelectric point, as judged by the turbidity of 1% gels at 2° C., was at pH 4.3 to 4.4.

RESULTS AND DISCUSSION

Effect of pH on Gelatin–Tannic Acid Reaction. pH is one of the more important environmental factors affecting precipitation in tannic acid–gelatin systems (Zitko and Rosik, 1962). Although Gustavson (1954) had shown that the binding of tannic by insoluble gelatins in the presence of excesses of tannin was independent of pH, when dealing with ordinary soluble gelatins and smaller amounts of tannin, a distinct pH optimum for gelatin and tannic acid precipitation can be seen (Figure 1), at pH 4.7 to 4.8, slightly below the isoelectric point, 5.2, of the calfskin gelatin used in these studies. It has not proved possible to determine the isoelectric point of the precipitated tannin-gelatin complex, but it is likely that the point had shifted toward the optimum pH found for precipitation. This could be due to a reaction between free amino groups of the gelatin and the few quinone groups found on tannic acid, thus decreasing the number of positive charges on the gelatin (Gustavson, 1966), and to an increase in the dissociation of the carboxyl groups at a given pH caused by stabilization of dissociated carboxyl groups through hydrogen bond formation with tannic acid hydroxyl groups.

As the pH went to the acid side of the optimum, the amount of tannin precipitated fell off sharply, so that R became lower. This indicated a decreased affinity between the gelatin and tannic acid. The greater amount of tannin and gelatin in the supernatant was probably due to a deaggregating effect of the gelatin's electrostatic charge, thus allowing more complex of a higher relative tannin content to remain in solution.

If so, greater gelatin precipitation together with lower R values at low pH levels would be expected when the isoelectric point of the gelatin was reduced. To check this conclusion some dinitrophenylated calfskin gelatin was prepared and its precipitation by tannic acid observed at different pH levels (Table I). The dinitrophenylation had the expected effect of increasing the amounts of tannin and gelatin precipitated at a given pH and also resulted in lower R_f values for the precipitates. Other consequences of dinitrophenylation, the addition of bulky side groups, the introduction of hydrogen-bondable nitro oxygens, and the decrease in the number of amino groups available for hydrogen bonding to peptide carbonyl groups, should have little effect on the aggregation of complexes to sedimentable size.

Table I. Precipitation of Calfskin Gelatin and Dinitrophenylated Gelatin by Tannic Acid at Different pH Levels

	pH					
	3.0	3.5	4.0	4.5	5.0	5.5
DNP gelatin						
% gelatin precipitated	40	63	93	97	95	76
% tannic acid precipitated	32	56	87	90	89	79
Unaltered calfskin gelatin						
% gelatin precipitated	20	45	69	78	76	47
% tannic acid precipitated	26	53	76	80	81	74

Systems had 50 mg. of gelatin and 25 mg. of tannic acid per 100 ml.

Because of the great effect of pH on the distribution of reactants and the need to carry out experiments where all the gelatin was precipitated without requiring huge excesses of tannin, a pH of 4.8 was selected for the rest of the work in this study.

Soluble Gelatin–Tannic Acid Complexes. Since it seemed probable that the soluble portion contained tannin combined to gelatin in a soluble complex, experimental proof was sought. For this purpose the elution patterns of gelatin and tannic acid from Biogel P-6 (CalBiochem) columns were examined and compared to the elution pattern of the soluble portion of a mixture of gelatin and tannic acid (Hummel and Dreyer, 1962). The solutions used were clarified by centrifugation and contained 0.6% gelatin and 0.2% tannic acid, alone or mixed.

The gelatin was eluted in the first five fractions, and the tannic acid appeared in the eighth and subsequent fractions. A broad peak for the tannin reflected its heterogeneity as well as a weak adsorption of the tannin on the Biogel. When the mixture of tannin and gelatin was applied, the tannic acid and the gelatin were eluted together in the same fractions as when gelatin alone was observed. This indicates a considerable stability for the soluble complex; otherwise a greater amount of tannin would have appeared in later fractions.

This stability of the tannin-gelatin complex raised the question as to the reversibility of complex formation. To test this, insoluble polyvinylpyrrolidone was utilized as a competitive binding agent for tannic acid by a procedure similar to Weiner and Koshland's (1965). A supernatant (20 ml.) from a mixture of gelatin and tannic acid (19 mg. of gelatin and 10 mg. of tannic acid) was added to 20 mg. of polyvinylpyrrolidone. After being shaken for 24 hours, the liquid phase was examined for gelatin and tannic acid. All of the original gelatin was found, but tannic acid was not detectable.

Very little information is available on the distribution of reactants between the supernatant and the precipitate where appreciable protein remains in solution (Page, 1942). Typical results obtainable with a range of reactant concentrations where partial precipitations occur (Table II) show that the amount of gelatin precipitation increased as the amount of tannin was raised. The tannin-gelatin ratio in the precipitate was often higher than that of the reactants at low reactant ratios. At high reactant ratios, as gelatin precipitation became more complete, the precipitate had lower ratios than the reactants. A greater percentage of the added tannin remained in solution at comparable reactant ratios when the amounts of added gelatin were low. This was also seen in dilution experiments described later.

As the amount of gelatin increased at a given tannin level, there was a leveling off of the amount of gelatin precipitated. This was checked in an experiment where the ratio of the reactants (grams of tannin/grams of gelatin) was lowered, by

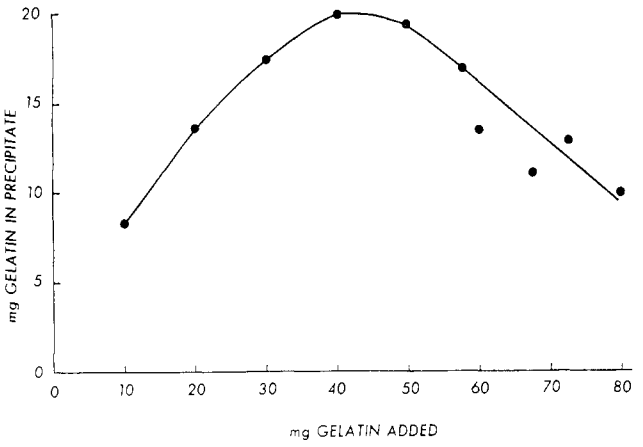

Figure 2. Variation in amount of gelatin precipitated under conditions of constant tannic acid concentration in 50-ml. volume reaction mixture (10 mg. of tannic acid per 50 ml.) and varying gelatin concentrations

pH 4.8

Table II. Ratios of Tannin to Protein of Supernatants and Precipitates in Systems of Varying Gelatin and Tannic Acid Concentrations

Added, Mg./100 Ml.		R'	R
Gelatin	Tannin	(Supernatant)	(Precipitate)
20	2	0.058	0.385
	4	0.097	0.464
	8	0.244	0.508
	16	3.840	0.607
40	4	0.047	0.400
	8	0.086	0.444
	16	0.195	0.542
	32	2.670	0.681
62	6.2	0.042	0.280
	12.4	0.082	0.367
	24.8	0.168	0.527
	49.6	3.03	0.704
82	8.2	0.036	0.346
	16.4	0.075	0.376
	32.8	0.190	0.493
	65.6	2.110	0.726
102	10.2	0.038	0.360
	20.4	0.072	0.409
	40.8	0.186	0.504
	81.6	1.550	0.741

stages, to 0.12, while the amount of tannins was kept constant (Figure 2). The maximum amount of gelatin was precipitated at a reactant ratio of 0.25. When more gelatin was present, the amount of gelatin precipitated decreased. Such decreases were presumably due to a lowering of the average weight of tannin bound per gelatin molecule below the level needed for extensive aggregation.

Tannic acid and gelatin are heterogeneous materials. Consequently, experimental results can be interpreted in various ways, depending upon the assumptions one makes as to the influence of the heterogeneity. One uncertainty involves the ability of the complete gelatin-tannin mixture to form insoluble complexes. A number of experiments showed that all the components of the gelatin could be precipitated by tannic acid. Tannic acid is not so simple, since one cannot secure complete precipitation of the tannic acid at any ratio of the reactants (Figures 1 and 3). Rather, there is a maximum

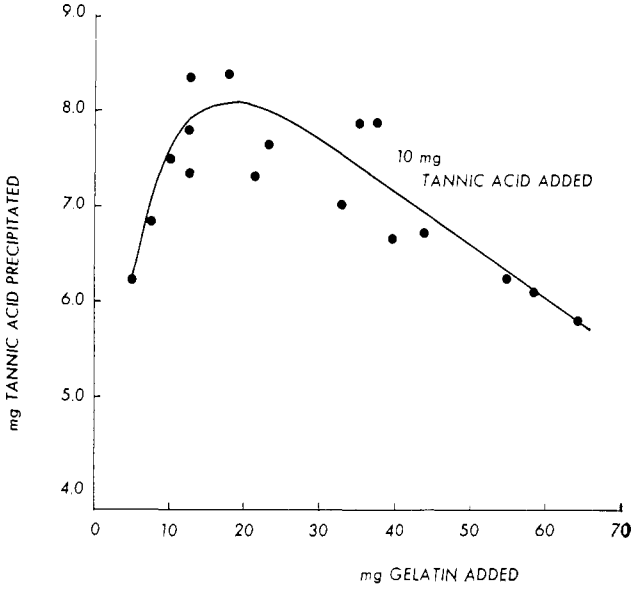

Figure 3. Variation in amount of tannic acid precipitated under conditions of constant (10 mg. per 50 ml.) concentration of tannic acid in reaction mixture and varying concentrations of gelatin

pH 4.8

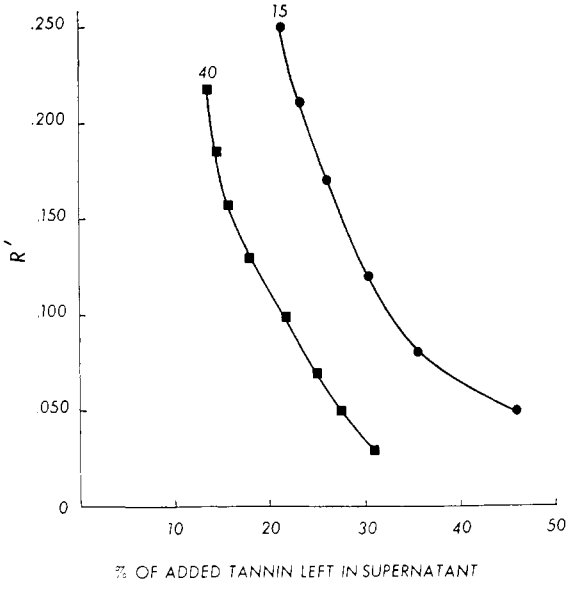

Figure 4. Decrease in tannic acid–gelatin ratio in supernatant as per cent original tannic acid left in supernatant decreased

Two gelatin levels tested, 40 and 15 mg./50 ml. pH 4.8. R'. Grams of tannic acid remaining in supernatant per gram of gelatin remaining in supernatant

precipitation when the reactant ratios are 1 part of tannic acid to 1 to 3 parts of gelatin.

One can check for nonprecipitating tannin by comparing the per cent of added tannin remaining in the supernatant with the ratio of tannin to gelatin in the supernatant, R' (Figure 4). The smaller the percentage of the added tannin left in the supernatant the greater the expected proportion of nonprecipitating tannins, hence the larger the R'. (This figure represents data obtained at reactant ratios where gelatin was present in larger concentrations than tannin—the opposite case would give a large R' value along with high percentages of tannin left in solution.) These results suggest that the tannic acid

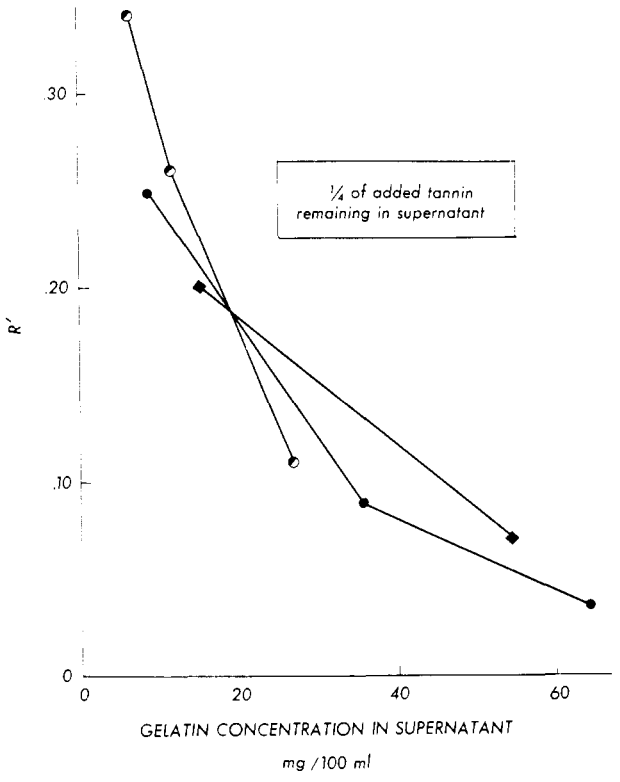

Figure 5. Change in R' with changes of gelatin in supernatant

Points plotted at constant percentage (25%) of added tannic acid remaining in supernatant. The three lines represent three different experimental series. pH 4.8
R'. Grams of tannic acid in supernatant per gram of gelatin in supernatant

preparation had in the order of 10% nonprecipitating tannin.

By using results obtained where a constant proportion of the added tannic acid remained in the soluble phase, some of the effects of tannin heterogeneity can be avoided. Of particular interest is the relation between R' and the concentration of gelatin in the supernatant. Interpolating the data from a number of experiments provides R' and gelatin concentrations corresponding to 25% of the added tannic acid remaining in the supernatant (Figure 5). Since it can be assumed that higher ratios of tannic acid to gelatin in the supernatant accompany higher degrees of binding of the tannic acid to the gelatin and the higher the degree of binding the greater the tendency to form precipitates, the increase of R' as gelatin concentration decreased shows that complex interaction leading to precipitate formation is very dependent upon the concentration of the complexes.

This conclusion was confirmed by a series of dilution experiments where the proportion of the reactants was kept constant over a wide range of concentrations. For these a ratio of 1 part of tannic acid to 4 parts of gelatin was used, since at such a ratio approximately equal amounts of soluble and insoluble complex would form. As seen in Figure 6, dilution had little effect on the per cent gelatin precipitated. At pH 4.7 there was a slight increase in that precipitated as the mixture was diluted 25-fold. At pH 4.0 there was a decrease. Such small changes should not be considered significant. At the three pH levels used, the per cent of tannin precipitated decreased (Figure 7) markedly at the 10-fold dilution. As further dilution took place, the curves leveled off. R went as low as 0.2 with 50% of the gelatin precipitated; the bound tannin responsible for insolubility did not leave the gelatin. In most cases of weak bonding, dilutions of five- to 10-fold

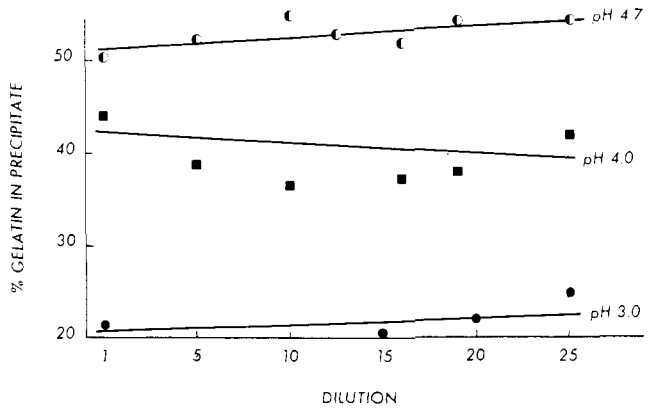

Figure 6. Effect of dilution on the % gelatin precipitated at three different pH levels

pH 4.7, 4.0, and 3.0. At 1 × dilution concentrations of reactants were 100 mg. of gelatin and 25 mg. of tannic acid per 100 ml.

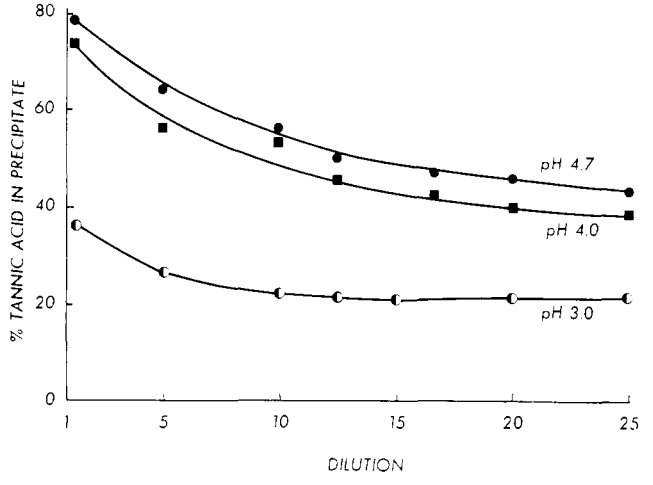

Figure 7. Effect of dilution on % tannic acid precipitated at three different pH levels

pH 4.7, 4.0, and 3.0. At 1 × dilution concentrations of reactants were 100 mg. of gelatin and 25 mg. of tannic acid per 100 ml.

greatly decrease the amount of bound material (Weber, 1965). The small amount of dissociation seen here, with less than half the tannin leaving the precipitated complex over a 25-fold dilution range, suggests multiple attachments of ligands. Undoubtedly, the tannic acid remaining on the insoluble gelatin was of a type forming especially strong bonds to the gelatin chains and having special abilities to cross link gelatin chains. The strength of the bonding is related to the large dipole moments (3.7 debyes) of the peptide groups directed approximately perpendicular to the chain axis (Meigham and Cole, 1964). With such large moments very stable hydrogen bonds can form, especially with polyhydroxy phenolic compounds that are bound at more than one site. At high dilutions the ratio of tannin to gelatin was nearly as great in the soluble portion as in the precipitated material. Since virtually all the soluble tannin is bound to gelatin, it is assumed that the insolubility of a tannin-gelatin complex is governed by other factors in addition to the number or weight of tannin molecules bound per gelatin molecule. One such consideration is the degree to which the hydrogen bond potential of a tannin molecule is used for bonds to a single peptide chain. The higher the extent of bonding on one chain the less able the tannin would be to form crosslinks with another chain. Large tannin molecules would be expected to bind to more than one

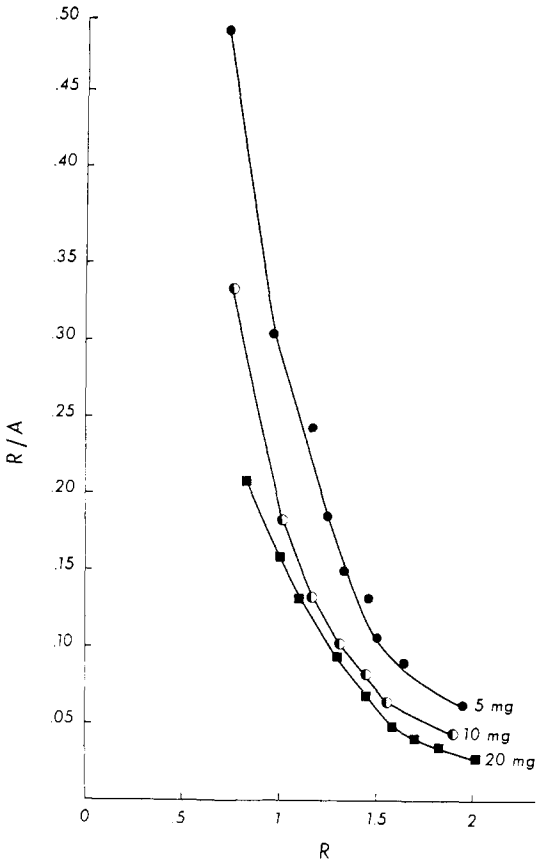

Figure 8. Plot of R/A *vs.* R for gelatin–tannic acid system

3 levels of gelatin plotted. 5, 10, and 20 mg. per 50 ml. pH 4.8
R. Grams tannic acid precipitated per gram of gelatin precipitated
A. Concentration (mg. per 50 ml.) of tannic acid in supernatant

gelatin chain. Rossi and Singleton (1966) showed that gelatin removed high molecular weight tannins from solutions in preference to low weight tannins. The results presented indicate a similar diversity in preference and reversibility.

As dilution increased, R' increased. Since any selective effect would imply that the tannic acid leaving the precipitate had a greater tendency to induce precipitation than the tannic acid fraction that had remained in the undiluted supernatant, it is again apparent that the lowering of the concentration of gelatin in the supernatant decreased the tendency of tannic acid–gelatin complexes to aggregate to sedimentable size.

Other examples of the reversibility of gelatin-tannin complexes have been demonstrated in the presence of urea and caffein (Mejbaum-Katzenellenbogen, 1959). Lowering of the pH also causes dissolution of insoluble complexes through electrostatic action. Addition of large excesses of gelatin act similarly by decreasing the R ratios of the complexes.

Binding Sites and Binding Constant. The determination of such parameters as the affinity between gelatin and tannin and the number of tannin binding sites per gram of gelatin would be useful in characterizing tannic acid–gelatin systems and the separate components. To obtain these values it is necessary to ascertain the uncombined tannic acid. Since the tannic acid–gelatin complexes normally consist of both soluble and insoluble components, one must work under conditions in which the soluble complex is isolated or eliminated. Equilibrium dialysis has been frequently used to obtain the concentration of free ligand when a small molecule and a large polymer interacted. Use of this method was not successful because of a decrease in the permeability of the cellulose dialysis tubing when exposed to the tannic acid–gelatin mixture. A similar result was found with cellulose nitrate. Furthermore, results on Biogel indicated very little uncomplexed tannic acid when significant amounts of soluble gelatin were in the system. An alternative method was to carry out the reaction under conditions where all the gelatin was precipitated; thus all the soluble tannic acid would be free tannic acid. Such conditions are found at concentrations of tannic acid equal to or greater than the concentration of gelatin and at pH 4.7 to 4.8.

The results of these experiments are plotted in Figure 8. The coordinates, $R \times 1/A$ (A = concentration of unbound tannin) and R, were chosen in order to express results in conjunction with the general theory of small molecule–large molecule interactions (Klotz, 1953). The equilibrium in such systems can be expressed by the equation $R/A = Kn - KR$, where K = equilibrium constant and n = maximum grams of tannin that can be bound by 1 gram of gelatin.

When this holds, the plot of Figure 8 allows the estimation of K as the intercept when $R = 0$, and of n when $R \times 1/A = 0$. Ideally, the plot should be a straight line, allowing confident extrapolation to the axes. Although Figure 8 shows that the data do not fit such a simple theory, some insight can be obtained by considering the reasons for the deviations. The theory assumes that all binding sites are equivalent and independent. With gelatin, made up of a mixture of amino acids, differences in the availability of sites are expected and thus give rise to a deviation from a straight line in the plot of experimental results. At low levels of R the most accessible sites with the higher K values are bound, thus giving the appearance of high K and low n for the system. As the amount of tannic acid increases, and R rises, there can be more and more binding at sites of low accessibility with an increase in the apparent n and a decrease in K. Another factor leading to an increase in n would be a reduction in the number of bonds joining a tannic acid molecule to the gelatin. In the case of a model tannic acid, such as pentagalloylglucose, there are 15 phenolic hydroxyl groups that might form hydrogen bonds to the gelatin peptide groups. With such a compound, all five galloyl residues can take part in hydrogen bonding with one peptide chain causing no bond angle strain, as can be seen through the use of Dreiding stereomodels (Rinco Instrument Co., Greenville, Ill.). With a molecular weight of 977 for pentagalloylglucose and an average amino acid residue weight of 73 for gelatin, one can have 2.7 grams of bound pentagalloylglucose per gram of gelatin if there is a perfect fit with only one hydrogen bond per galloyl residue. Furthermore, in systems where a multifunctional small molecule binds to a much larger polymer, the fractional saturation of the polymeric binding sites reaches a practical limit at 0.8 (Latt and Sober, 1967). This would give a value of 2.16 for n. This is close to the n value that would be obtained if the points obtained at the lower R values were extrapolated to the R axis. Of course, the flattening of the curves at high R values suggests that fewer than five peptide binding sites are being used. In fact, a plot of R/A *vs.* $1/R$ indicates that a maximum value of nearly 4 can be expected for n. A value this high signifies that the tannic acid, considered as a pentagalloylglucose, reacts at between two and three peptide sites of the gelatin molecule when the tannic acid concentration is high. The final limit would be one tannic acid molecule for each peptide site.

Another point brought out in Figure 8 is that the curves are dependent upon the initial concentration of the gelatin,

because, at a given R, there was a larger measured value for A as the amount of gelatin increased. This behavior is further evidence that the tannin-gelatin reaction cannot be adequately described by mathematical treatments of binding equilibrium which assume the independence of polymer concentration with regard to the estimation of K and n values.

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