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I hereby recommend that the thesis prepared under my supervision by Herbert C Stecker *entitled* A Study of the Combination of p-Benzoquinone with Collagen

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

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A Study of the Combination of p-Benzoquinone with Collagen

A Thesis

by

Herbert C. Stecker

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A Study of the Combination of p-Benzoquinone with Collagen

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Introduction:

The remarkable tanning properties of p-benzoquinone, first pointed out by Meunier and Seyewetz (22), have been known for many years. In spite of the undeniably valuable properties possessed by this tanning agent, however, other factors such as difficulty of handling, expense and low weight yield in the tanned hide, have militated against its commercial adoption and it has remained only a scientific curiosity. From the standpoint of the fundamental investigation of the mechanism of tanning, however, quinone is important for at least two reasons. These are the high degree of water resistance imparted by it in tannage, and the fact that the tanning agent, at least as it is initially used, is a well-defined chemical entity of relatively low molecular weight and known structure. Experience in the investigation of formaldehyde tannage has suggested that under the latter conditions a great advantage is gained in the working out of possible stoichiometrical relations and the undertaking of the tanning reaction.

Moreover, in order to fully understand this benzoquinone-collagen reaction, it is most vital to know the exact nature, not only of the initial reagent, but also

of the final reacting substance because in no other way is it possible to predict the actual combination mechanisms and account for the phenomena observed.

History:

In 1924 Thomas and Kelly carried out a series of experiments in which they determined the effect of the pH value of the solution on the fixation of quinone by hide powder. They also studied the effects of other factors. This was followed in 1926 by further work by Thomas and Kelly (34), and by Thomas and Foster (32), who studied the effect of deaminization of the collagen on quinone fixation. Aside from these, and the earlier work of Meunier (21) and his collaborators, no other investigations of the reaction between quinone and collagen appear to have been recorded.

In the work of Thomas and Kelly, referred to above, a characteristic and striking minimum point of quinone fixation was found, which they located at pH 9.0. At the time this work was carried on, however, there was no method of determining the pH value of a solution containing quinone, the hydrogen and quinhydrone electrodes both being inapplicable. Their method of work, therefore, was to dissolve the quinone in phosphate buffer solutions of known pH values, and to use these values in the construction of the curves representing their results. It is

clear that changes of pH caused by the solution of the quinone, and by absorption of the components of the buffer mixture by the hide powder, might lead to a decidedly different shape of curve, or at least to a shift in its position, when the true equilibrium pH values are used instead of the initial pH value of the buffer mixture. Since preliminary experiments indicated that the pH changes caused by these factors were quite considerable, it was decided to initiate the present investigation by repeating the work of Thomas and Kelly, making measurements of the true pH values at tanning equilibrium by means of the glass electrode, which permits determinations in solutions of quinone.

Experimental Methods:

1. Tanning with Quinone.

In checking the previously reported work, the procedure of Thomas and Kelly (33) was followed in detail with the exception that 0.1M buffer solutions were used in all possible cases. Saturated solutions of pure quinone were prepared by dissolving 2.74 grams of the compound in 200 mls. of buffer solution. To these portions of tanning solutions were added 2.000 grams of dry, ash-free collagen powder (15), or its equivalent. (In the case of tanning hide powder, 2.000 grams of dry, ash-free standard hide powder was used.)

Tannage was carried out at room temperature with intermittent shaking, except in 6 and 24 hour tanning periods, when shaking was continuous. At the end of the desired tanning period, the pH values of the liquors were determined on the glass electrode, after which the tanned samples were washed with distilled water in Wilson-Kern extractors until the wash waters were perfectly colorless and gave a negative starch-iodide test for quinone. This test is claimed to be able to detect 1 part of quinone in 50,000 parts of water. The time and volume of water required to wash the tanned samples varied considerably with the conditions of tannage, but in no case was less than 5 liters employed.

After washing, the samples were removed from the extractors and allowed to air-dry, subsequently to be dried in vacuo at 110° C. for 16 hours. The increase in weight is taken to be the amount of quinone fixed by the collagen or hide powder.

The periods of tannage were arbitrarily set at 6 hours, 24 hours, 1 week, 2 weeks, and 5 weeks, to conform approximately to those employed by Thomas and Kelly.

2. The Preparation of a Quinone Polymer.

To an alkaline aqueous saturated solution of quinone is added approximately a hundredth of a part by weight of formic acid (the mixture must be made just acid

to litmus). This mixture is then warmed gently on the steam bath for an hour to aid coagulation and precipitation of the insoluble portion. It is cooled and the supernatant liquid is centrifuged from the solid matter. The residue is finally drained of excess moisture through the use of a good filter paper, after which it is dried on the steam bath in an open drying dish. For a very pure product, the dried material is washed with distilled water to which has been added a small amount of formic acid. The final product is a shiny, brownish-black solid, having no melting point and a high ignition temperature; on ignition it burns with a sooty, luminous flame. The yield is nearly quantitative.

3. Tanning with the Polymer of Quinone.

The same procedure was followed as that above for quinone tannage, with the exception that weight allowances were made in the case of the polymer so that, as nearly as the assumptions are correct, the same number of potential quinone units are present as above.

4. Molecular Weight Determination Methods.

Due to the limited solubility and the general properties in all of the solvents tried, it was not found possible to attempt molecular weight determinations of the quinone polymer by the ebullioscopic, cryoscopic, vaporimetric or viscosimetric methods with any degree of accuracy.

An osmotic method has been evolved, however, from the original method of Barger (2,13,14,27), in which the capillary tubes are alternately filled with several droplets of the standard and sample solutions, and the diameter of the droplets is measured. This method is based upon the isothermic distillation in a closed system of solutions of unequal molar concentrations but identical solvent. The osmotically weaker solution, of lower molar concentration, gives off so much from the volatile solvent to the osmotically stronger solution, of high molar concentration, until equilibrium is reached. When no change in the diameter of the droplets is observed the molar concentration of the two solutions must be the same.

A modification of the osmotic method was also used in the form of an isothermic distillation method (4,29). In this method an apparatus was constructed which allowed a free passage of solvent vapors between a solution of azobenzene standard and a solution of the polymer, each of known concentration. The progress of the low temperature vacuum distillation was observed by measuring the volume of the two solutions by means of calibrated side-arms attached to the bulb of each distillation arm. When the system reached equilibrium the solutions were at equal molar concentration. According to Raoult's Law, it then becomes a simple mathematical procedure to determine the

molecular weight of the unknown substance.

$$M_1 = \frac{M G_1 V}{G V_1}$$

where M, G, and V represent respectively molecular weight, grams of solute and volume of solution of the standard, and M_1, G_1, and V_1, refer to corresponding data with regard to the unknown substance.

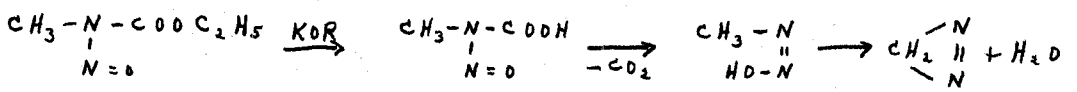
Having exhausted all feasible methods of molecular weight determination at this laboratory, it was deemed advisable to attempt a chemical check on the physically determined data. Assuming that a measurement of the number of hydroxyl groups would indicate a possible size or configuration of the polymeric substance, several quantitative estimations involving those groups were conducted.

In the first of these estimations the hydroxyl number was found by the Roberts-Schuette acetic anhydride method (26). In this procedure the sample is introduced into a tared tube and weighed, after which an appropriate quantity of acetic anhydride is weighed into the tube in the same manner. The tube is sealed and placed in an oven at 120° C. for an hour, with occasional shaking. It is then cooled, carefully opened into a 500 ml. Erlenmeyer flask containing 50 ml. of water, rinsed with cold and hot water, and made up to about 200 ml. To the contents of the

flask are added a known amount of KOH and a few glass beads, and the mixture is brought almost to a boil under a reflux condenser. After cooling and detaching from the condenser the mixture is titrated with standard KOH to a thymol blue end-point. The hydroxyl number, defined as the number of milligrams of KOH equivalent to the hydroxyl content of one gram of sample, is given by the formula:

$$OH^{\#} = \frac{\text{mg. KOH involved}}{\text{weight of sample}}$$

The second of these estimations involved the methylation of the polymer with diazomethane, and subsequent methoxy group determination of the methylated sample. The diazomethane was freshly prepared for use by the method of von Pechmann (24). This procedure is merely the formation of diazomethane from nitrosomethylurethane by the action of methyl alcoholic KOH:



Ether and heat are added to carry the product over into ice-cold ether for temporary storage without danger of explosion.

The third type of estimation was conducted for a two-fold purpose; first, to check the diazomethane methylation procedure and, second, to find whether all or part of the hydroxyl groups were acidic. Since it is a fact that

the rate of reaction of diazomethane increases with the acidity of the hydroxyl group which is being methylated, it is possible that some of the hydroxyl groups of the compound, if alcoholic, did not react with the reagent. By using dimethyl sulfate (which reacts with all hydroxyl groups) as the methylating agent, one of two things may be decided from the data:

(1) If the methoxy group determination of the diazomethane and dimethyl sulfate reaction products are identical, all of the hydroxyl groups are at least moderately acidic in nature;

(2) If the two methods of methylation do not give the same results, it is very possible that there are a certain number of alcoholic hydroxyl groups in the molecule and it is not a uniformly reactive structure. The ratio of the alcoholic to the acidic hydroxyl groups can be calculated.

The methoxy group estimation was performed on a semimicroquantitative scale (35,36,41). The principle of the determination of alkoxy groups is that when an organic compound containing one or more of these groups is refluxed with hydriodic acid and suitable aiding chemicals, the alkoxy radical is split off in the form of the corresponding alkyl iodide:

$$R{-}O{-}R' + 2HI \longrightarrow 2RI + H_2O$$

$$\phi{-}O{-}R + HI \longrightarrow \phi{-}O{-}H + RI$$

$$>N{-}R + HI \longrightarrow >\underset{\underset{H\ I}{|}}{N}{-}R \xrightarrow{\Delta} >N{-}H + RI$$

The phenol is added as a catalyst, and the acetic anhydride as a solvent for the sample. The alkyl iodide formed in the refluxing chamber is absorbed in a solution of sodium acetate in acetic acid to which a few drops of bromine have been added:

$$RI + Br_2 \longrightarrow RBr + IBr \; ; \qquad IBr + 3H_2O + 2Br_2 \longrightarrow HIO_3 + 5HBr$$

The sodium acetate neutralizes the hydrobromic acid formed:

$$HBr + CH_3COONa \longrightarrow NaBr + CH_3COOH$$

$$CH_3COOH \rightleftharpoons [H^+] + [CH_3COO^-]$$

A washer inserted between the refluxing chamber and the absorption tube is charged with 5% sodium thiosulfate solution to absorb any hydriodic acid that might have refluxed over and would otherwise interfere with the analysis:

$$2HI + Na_2S_2O_3 \longrightarrow 2NaI + H_2O + SO_2 + S$$

The excess bromine in the absorption tube is decolorized with formic acid:

$$Br_2 + HCOOH \longrightarrow 2HBr + CO_2$$

The iodine liberated after the addition of potassium iodide is titrated with 0.01N solution thiosulfate:

$$2HIO_3 + 10KI + 5H_2SO_4 \longrightarrow 6I_2 + 5K_2SO_4 + 6H_2O$$

$$I_2 + 2Na_2S_2O_3 \longrightarrow 2NaI + Na_2S_4O_6$$

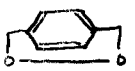
5. Particle Size Determination.

Since the phosphate polymer in most penetration experiments diffused into the hide very difficultly, it was reasoned that this phenomenon might be explained on the basis of particle size. On a microscopic examination of polymer-tanned collagen, washed so as to remove all discernable particles occluding to the fibers, the intense color of the polymer could be detected very easily and yet no particles could be found. Ruling out the theory that the molecular size of the polymer was such as to prevent penetration, there remains the possibility that the particle size of the polymer is the explanation of the fact.

The particle size was determined by the applications of the ultrafiltration principle. A series of collodion membranes was made up, having average pore diameters ranging from 5 to 318 millimicrons. By filtering the polymer "solution" through a number of such membranes with the aid of 25 lbs./in.^{2} pressure of nitrogen, various percentages of the original concentration of the "solution" were obtained. By plotting percentage of original concentration against average pore diameter, it was possible to obtain a curve from which the maximum particle size of the polymer may be ascertained.

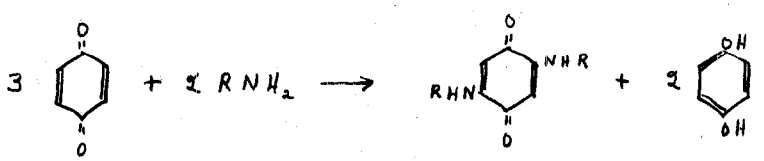
The Action of Quinone and α-Amino Acids:

A considerable amount of investigation has been conducted regarding the reaction between quinone and α-Amino acid, but the conflicting reports only succeed in adding confusion to the problem. For example, according to Wieland (38) the action of quinone is oxidative, due to the fact that it removes hydrogen directly from an oxidizable, hydrogen-containing substance and forms hydroquinone. Bach and Nikolaev (1) have shown that the concept is not accurate, since the reaction does not proceed in the absence of water. They propose that quinone acts as an oxidizing agent, not because it takes hydrogen from an oxidizable substance to form hydroquinone, but because, in its peroxide form:



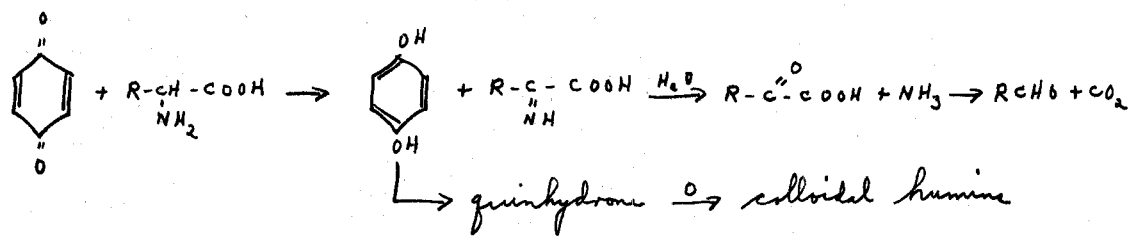
it adds water and then, as p-hydroxyphenyl hydroperoxide:

gives off its active oxygen to the oxidizable substance and is itself deoxidized to hydroquinone. But, whatever the oxidation mechanism may be, Fischer and Schrader (12) have stated the reaction to be:



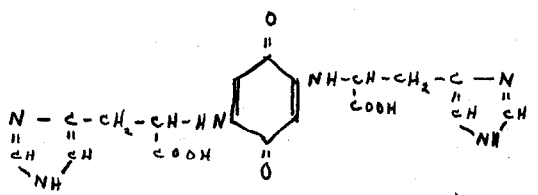
In direct opposition to this theory, Moeller (23) has reasoned that there must be two classes of quinone:

(1) Those having a quite stable quinone character which polymerize, forming colloidal complexes, and (2) those unstable in water which oxidize α-Amino acids to aldehydes, forming colloidal complexes as a result:

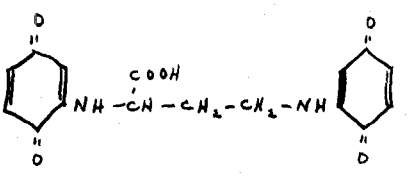


Kav (19) has stated in this connection that all quinones cause deamination of most of the amino acids, without catalysts or oxidizing agents.

The work of Woker and Antener (40) has been an attempt to incorporate both views concerning the reaction. They suggest that mono-amino acids form o-di- (amino acid) quinone, and that diamino acids employ two quinone molecules:

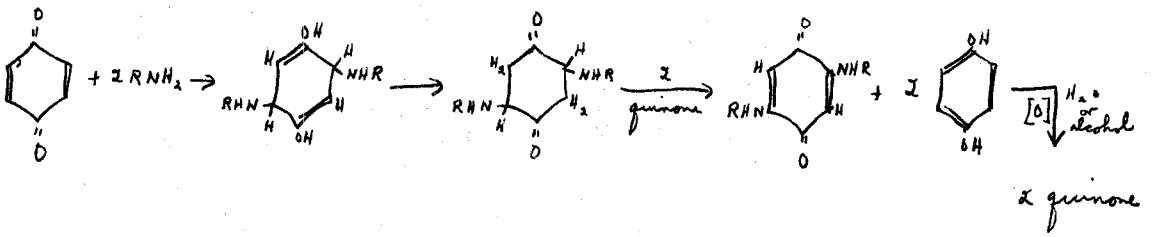


(di-histidin-quinone)



(ornithin-dijuinone)

The mechanism of these formations have been worked out by Cooper and Haines (5,6):

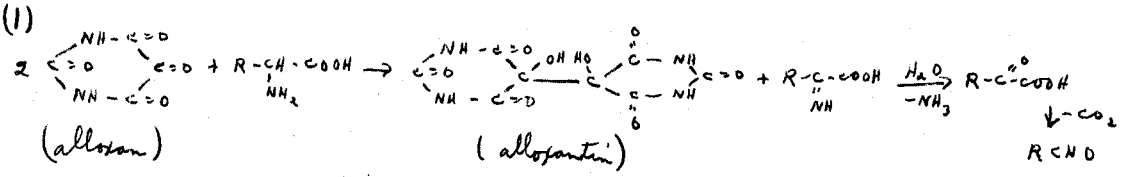


Woker and Antener go on to suggest that probable secondary reactions are:

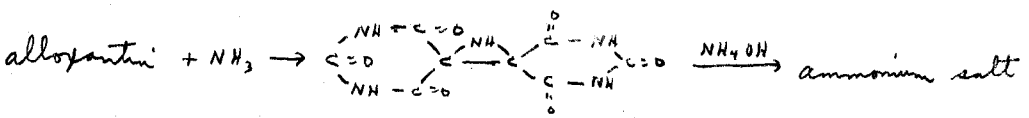
(1) Deamination of the amino acid (by analogy with Strecker's reaction between alloxan and amino acids (30), and the reaction between ninhydrin and amino acids) to form hydroquinone, ammonia, carbon dioxide and an aldehyde with one less carbon atom. (See Moeller's reaction above)

(Strecker's reaction)

(1)

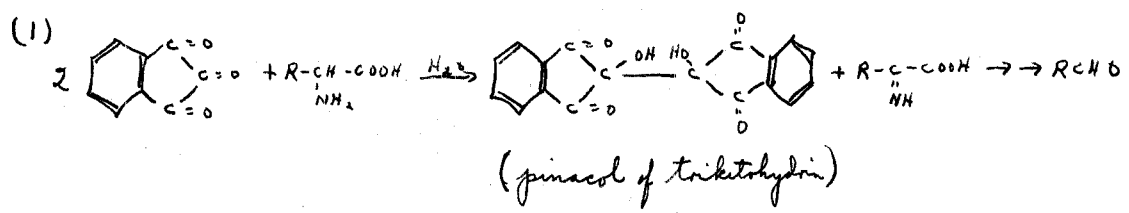


(2)

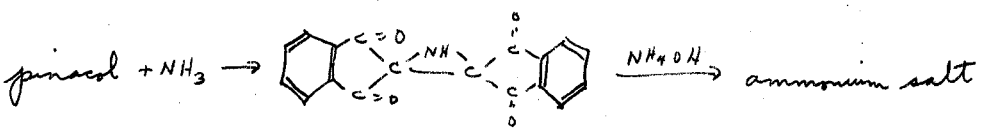


(Reaction between ninhydrin and amino acids)

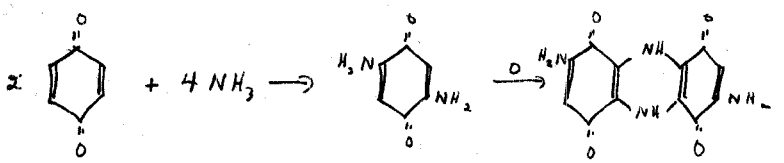
(1)



(1)

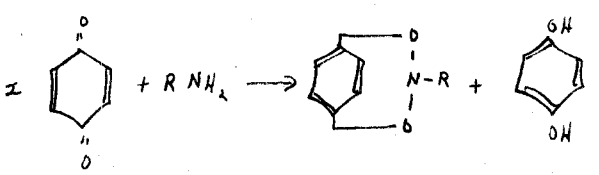


and (2), further action involves the formation of quinonediamine from the liberated ammonia which, on oxidation, yields condensed ring systems:

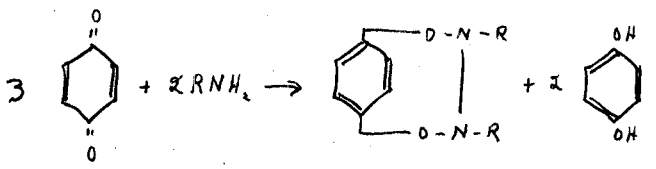


The Action of Quinone on Protein.

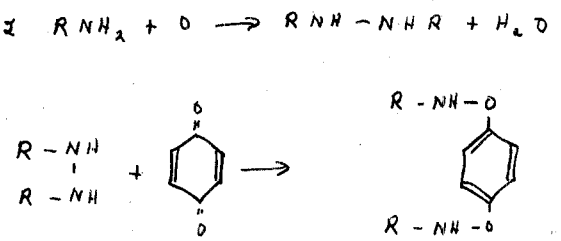
The foregoing theories concerning the reaction of quinone with α-amino acids may be adapted quite readily to the study of the protein-quinone reaction. It is very likely that quinone is able to react with the free amino groups of the protein and account in part for the tannage phenomenon. This mechanism has been suggested by Meunier (21) as,



and,

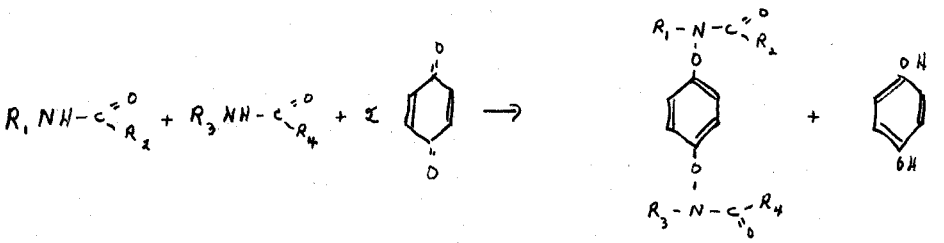


and also by Fahrion (14,15) as,



but previously mentioned interpretations have been accorded more weight.

Wilson (39) has extended the action of quinone to the backbone structure of the protein, and thus revealed a method of utilizing a much larger number of quinone molecules per protein molecule:

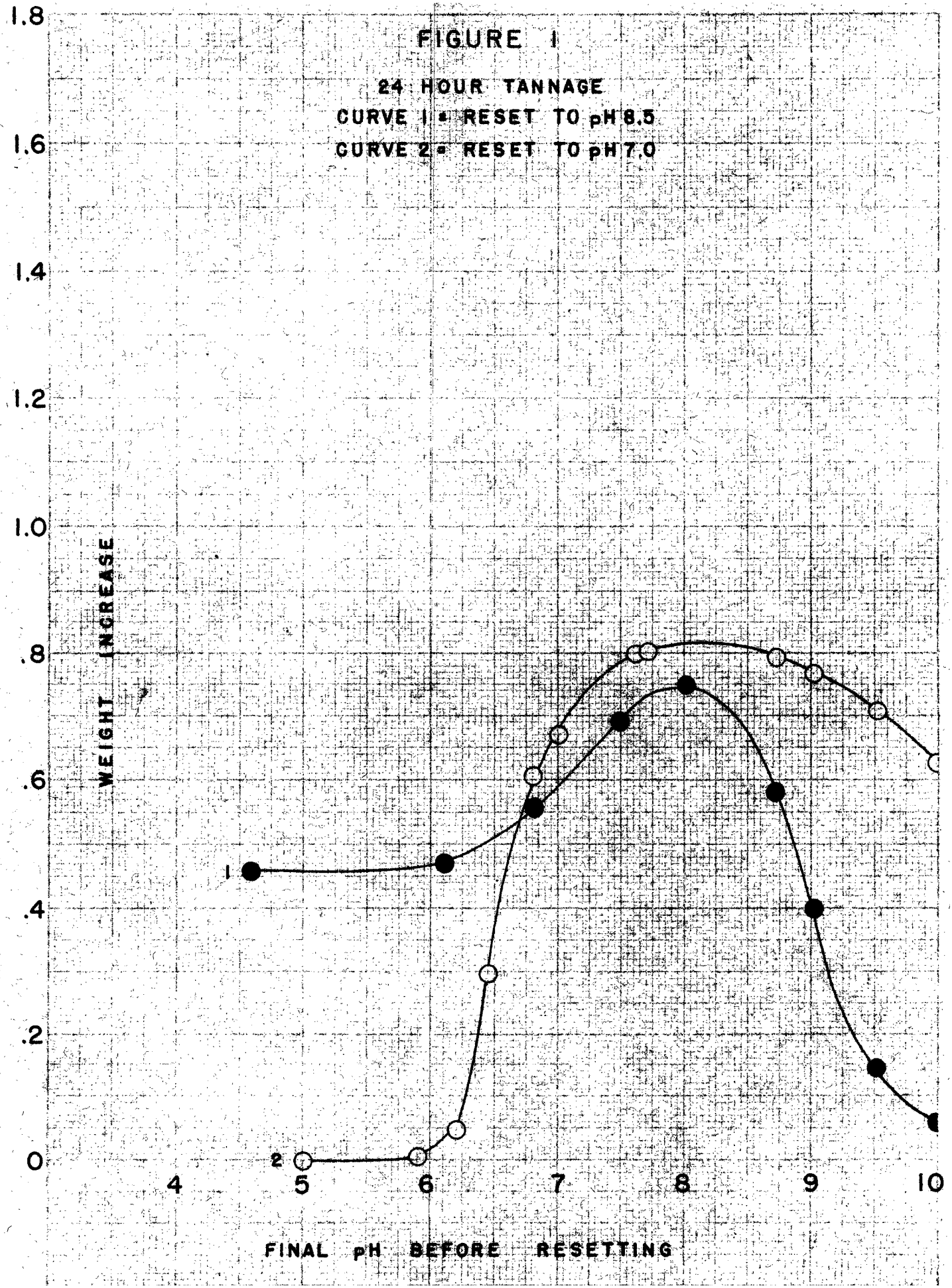


His hypothesis has suggested an explanation for the action of quinone upon deaminized collagen (18).

These reactions perhaps constitute the principal action of quinone as such on the protein molecule. But, to extend the action to the physical reasons for weight increase. Cooper and Haines, et al (5,6,31,37)

have reported that adsorption was a large factor. In experiments involving the use of gelatin, they discovered that quinone may be adsorbed in ever-increasing quantities for at least three months. (Cooper and Nicholas (7) in previous work showed that the action was evidently primarily adsorption, because of the similarity of action between p-benzoquinone and its methyl derivative). The chemical changes in quinone solutions were listed as oxidation, reduction and polymerization, but the last was not further explained.

It may be reasoned in this connection that if a polymer can be formed in solution, it can also be formed in the interstices of, and perhaps actually within, the collagen fibers. If this were the case, the larger molecule or the particle formed would be inescapably imbedded in the material and would produce a weight increase curve. An experiment was conducted in which samples of collagen were tanned with quinone for twenty-four hours over the usual pH range and then all of the samples were made alkaline (pH = 8.5); tannage was again allowed to proceed for twenty-four hours, after which the samples were washed and dried, and this weight increase reported. Figure I, Curve I, indicates that the polymerization is likely to take place. In addition to this experiment, samples of collagen were tanned under the same conditions as before, except



that instead of the pH being reset to 8.5, it was reset to 7.0. As can be expected from the limited solubility of the polymer in neutral or acid solution, Figure I, Curve 2, shows an indication of its precipitation in and around the fibers. The formation and deposition of this polymer in the fiber or fibril may be the reason for the marked similarity in the X-ray diffraction diagrams of vegetable and quinone tanned collagen (17,18), since the change is apparently dependant upon the molecular or particle size of the "tannin."

The Effect of the Addition of Hydroquinone on Quinone Tannage.

According to previous investigators (28,33,34), the presence of hydroquinone in a quinone tanning solution represses the amount of tannage. The results of this investigation have verified that phenomenon, as may be seen with corrected pH values by reference to Figure 2. An interesting side-light on the subject was suggested by Buchholez (3), who pointed out that hydroquinone salts gelatin out of solution; this may indicate that a salting-out process, in repressing adsorption, thus decreases the weight value by which means the degree of tannage is measured.

However, perhaps the most important reason for this repressing influence of hydroquinone on quinone tan-

FIGURE 2

EFFECT OF HYDROQUINONE ON TANNAGE

● = 24 HOUR TANNAGE (pH 6.35); ◑ = 24 HOURS (pH 7.75)

◐ = 2 WEEK TANNAGE (pH 5.55); ○ = 2 WEEKS (pH 7.20)

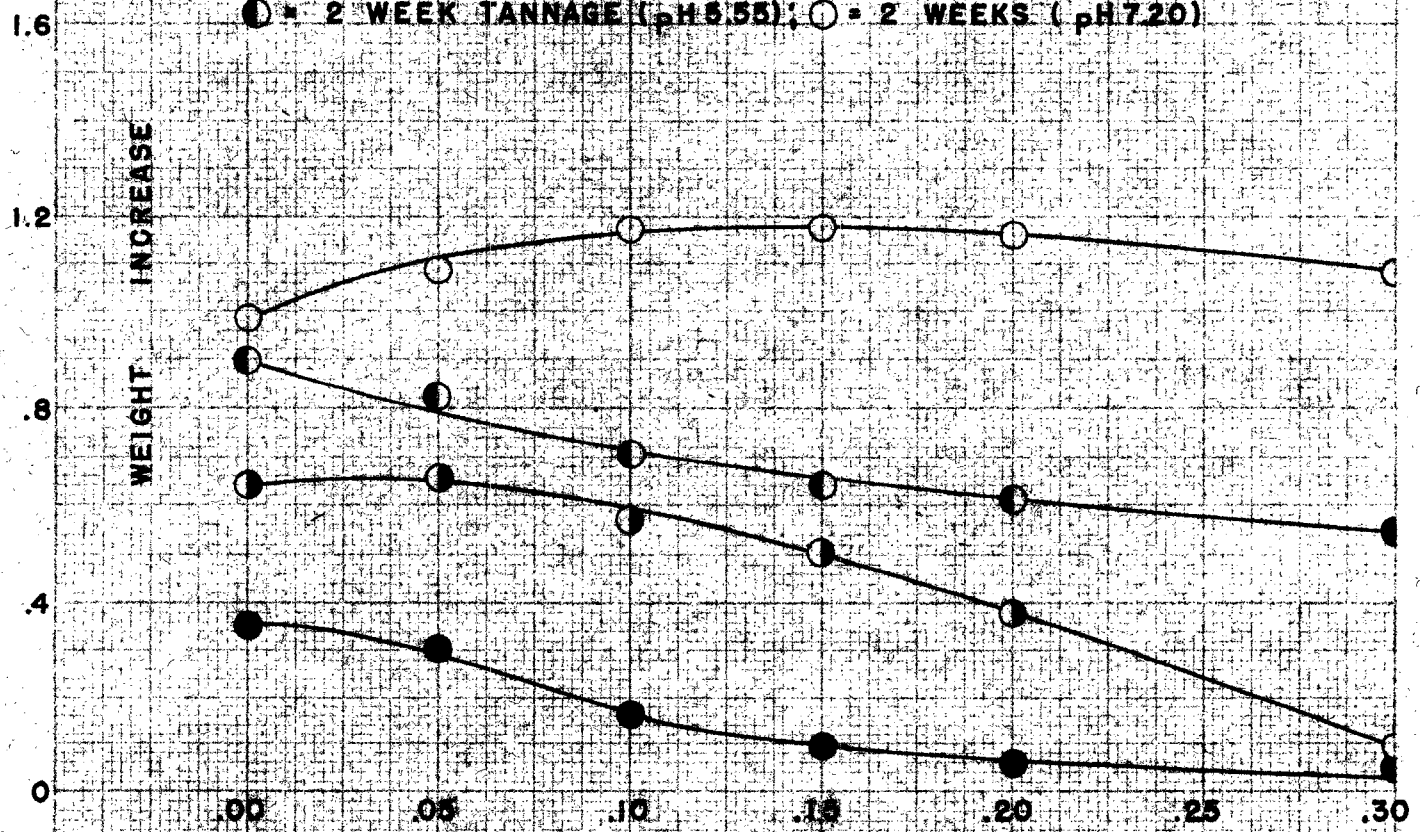
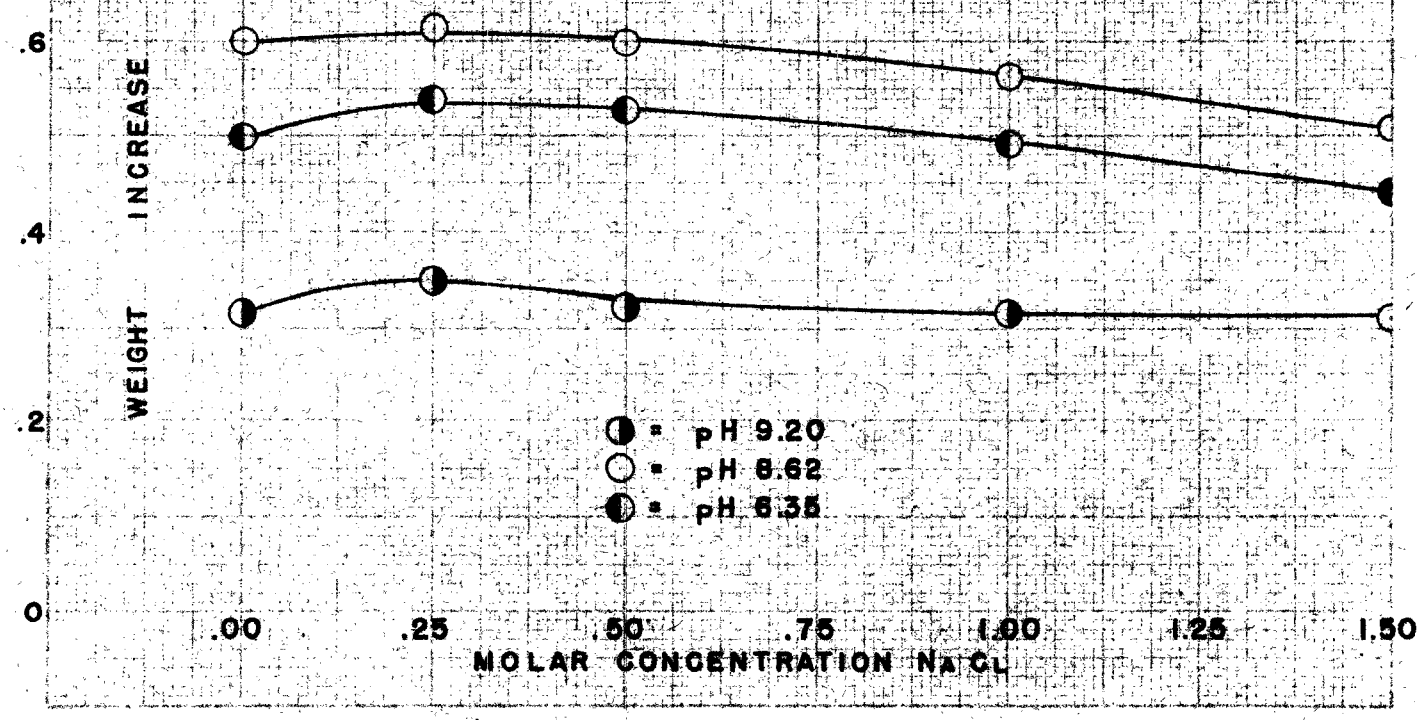


FIGURE 3

EFFECT OF $Na\,Cl$ ON TANNAGE (24 HOURS)



nage may be shown by reference to most of the theories of the quinone-protein reaction (21). It will be noted that one of the by-products of the reaction is hydroquinone and, by the application of the law of mass action, the addition of more hydroquinone to the solution should repress the reaction.

The Effect of the Addition of Sodium Chloride on Quinone Tannage.

Thomas and Kelly (34) have shown that the addition of sodium chloride progressively decreased the rate of tanning, except in low concentration, where it is slightly increased. The results of this investigation has confirmed their work and supplied the corrected pH values. (See Figure 3).

RESULTS AND DISCUSSION

Tanning in Phosphate Buffers.

The weight increase of collagen powder in quinone phosphate buffer systems, as a function of pH and of time, is tabulated in Table I, and shown graphically in Figure 4. In these curves the presence of the minimum point is definitely confirmed and its location on the pH scale has been revised according to the measurements made possible by the glass electrode. The true pH of the minimum point is seen to be 7.5. Measurements of the initial pH values of the buffer solution indicate that these data check well with those of Thomas and Kelly.

It will be noted that the minimum point on the curves falls very near the isoelectric point of the collagen used, which is about pH 7.8 (16). This suggested the possibility that the minimum point might be due to, or related to, the isoelectric point of the collagen. In order to test this possibility, the experiments were repeated, using standard hide powder, the isoelectric point of which is close to pH 5. With the exception of this change in the material being tanned, the procedure was identical with that used before. The results obtained are shown in Table II and Figure 5. If the minimum point is related to the isoelectric point of the collagen, it would be expected that in the case of the standard hide

powder a shift would occur to a lower pH value. Inspection of Table II and Figure 5 shows, however, that such is not the case. The minimum point is again found at pH 7.5, and consequently it must be concluded that it is not related to the isoelectric point of the collagen.

TABLE I

Weight Increase of Collagen Powder
in Phosphate Buffered Quinone Solution

(2,000 gms. Collagen in 200 ml. Solution)

Initial pH	Final pH	Weight Increase	Initial pH	Final pH	Weight Increase
	(6 hours)			(24 hours)	
5.0	5.0	-.020	5.0	5.0	.042
6.0	5.9	+.055	6.0	5.9	.100
7.0	6.8	.280	6.5	6.4	.500
8.0	7.3	.440	7.0	6.8	.658
9.0	7.5	.425	8.0	7.3	.662
10.0	7.6	.450	9.0	7.5	.640
10.5	8.0	.445	10.0	7.6	.665
10.8	8.7	.320	10.5	8.0	.700
11.0	9.0	.242	10.8	8.7	.585
11.6	9.5	.100	11.0	8.9	.405
			11.6	9.5	.150
			12.0	10.0	.050
	(48 hours)			(1 week)	
5.0	5.0	.100	5.0	4.6	.450
6.0	5.9	.210	6.0	5.9	.775
7.0	6.8	.838	7.0	6.7	.950
8.0	7.3	.840	8.0	7.3	.965
9.0	7.5	.805	9.0	7.5	.880
10.0	7.55	.807	10.0	7.6	.982
10.5	8.0	.870	10.5	8.0	1.015
10.8	8.7	.750	10.7	8.5	.915
11.0	8.9	.520	10.8	8.7	.700
11.6	9.5	.150	11.0	8.9	.410
			11.6	9.5	.150
			12.0	10.0	.050
	(2 weeks)			(5 weeks)	
5.0	4.1	.998	5.0	4.0	1.160
6.0	5.6	.910	6.0	5.55	1.075
7.0	6.8	1.002	7.0	6.7	1.045
8.0	7.3	.968	8.0	7.2	1.005
9.0	7.4	.885	9.0	7.4	.925
10.0	7.5	.880	10.0	7.5	.902
10.5	8.0	1.012	10.5	8.0	1.015
10.8	8.7	.925	10.8	8.65	.945
11.0	8.9	.520	11.0	8.9	.520
11.6	9.5	.150	11.6	9.5	.150

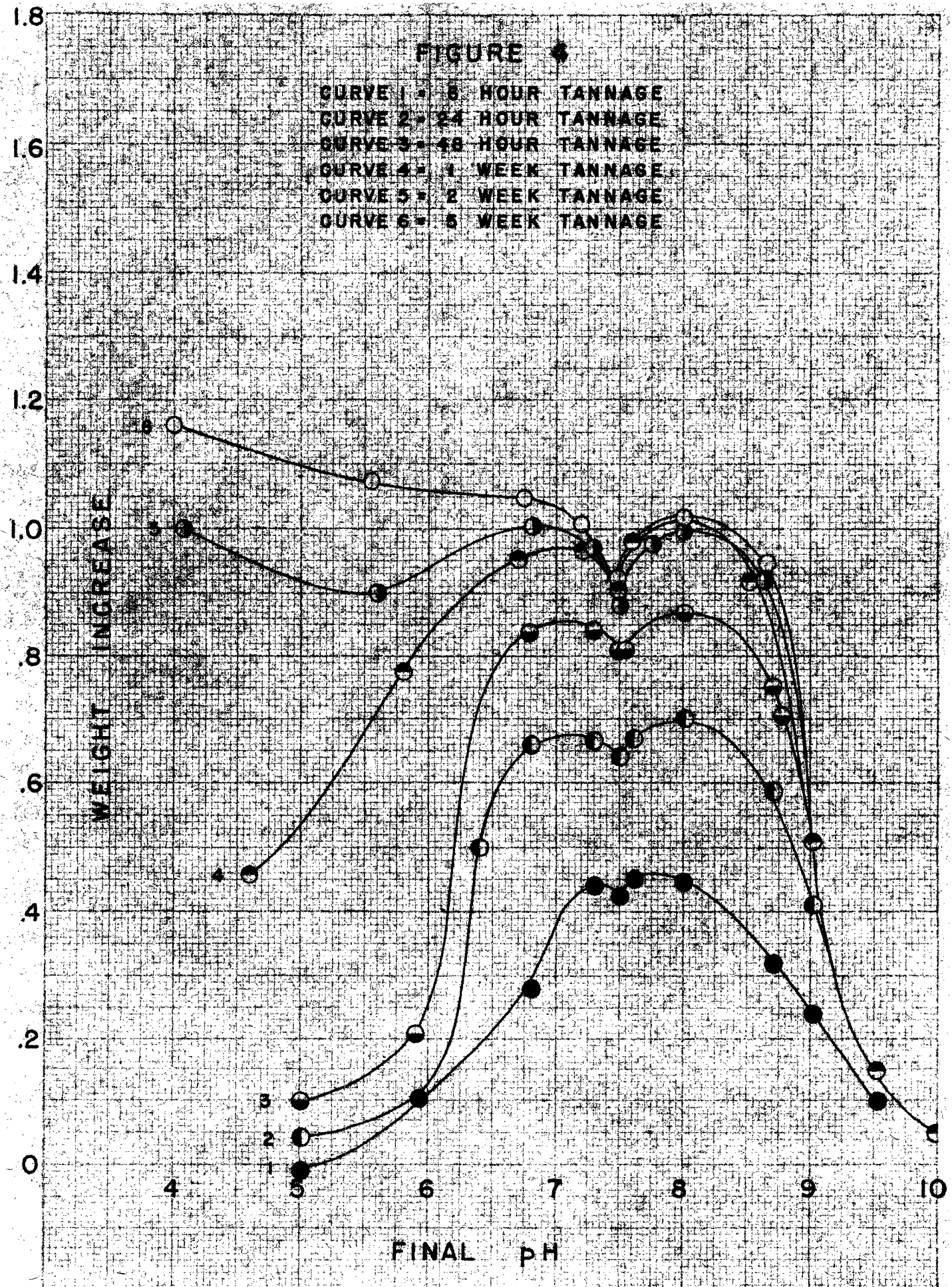
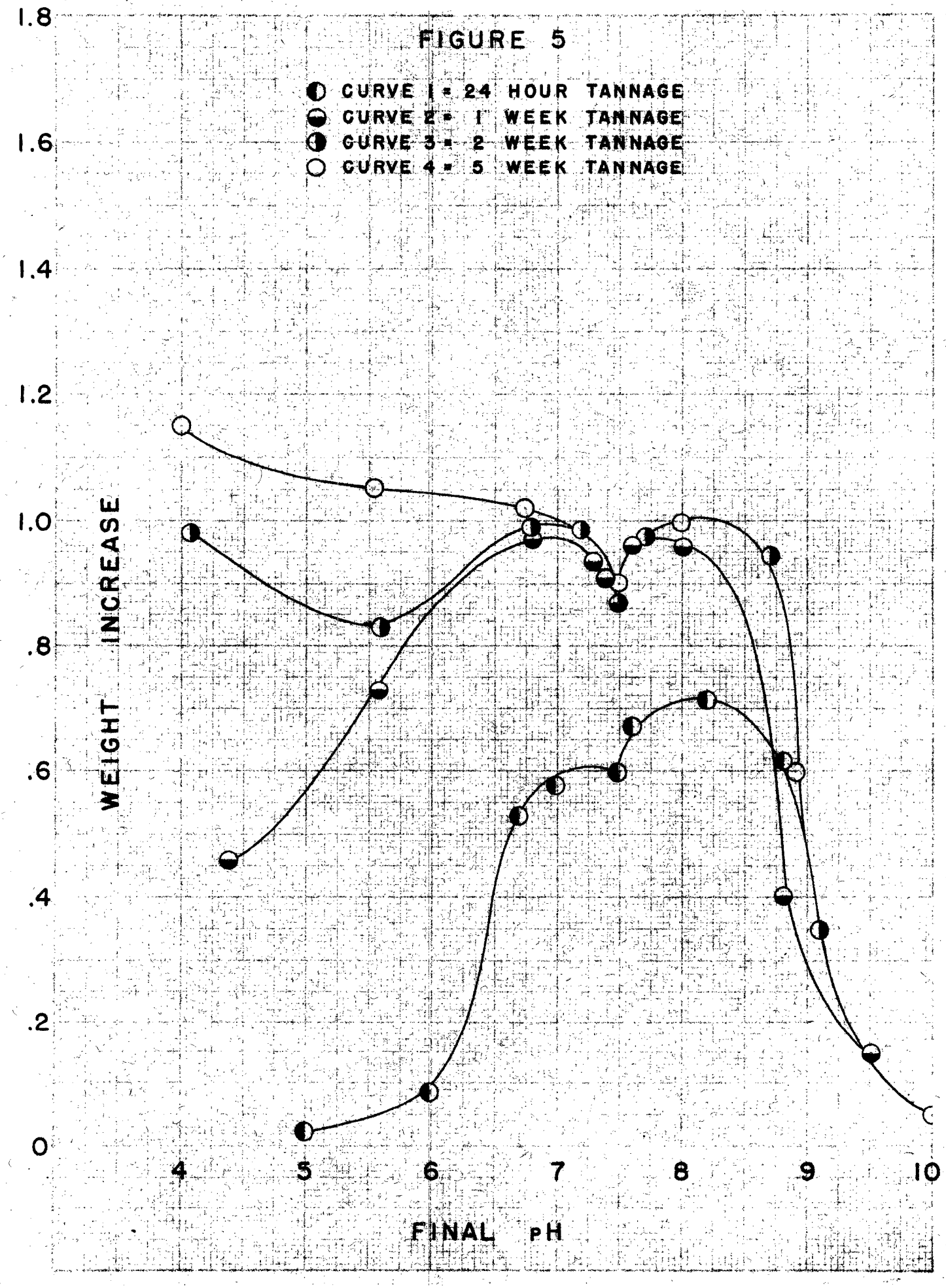


TABLE II

Weight Increase of Hide Powder
in Phosphate Buffered Quinone Solution

(2,000 gms. Hide Powder in 200 ml. Solution)

Initial pH	Final pH	Weight Increase	Initial pH	Final pH	Weight Increase
	(24 hours)			(1 week)	
5.0	5.0	.021	5.0	4.4	.460
6.0	6.0	.086	6.0	5.6	.730
7.0	6.7	.530	7.0	6.8	.972
8.0	7.0	.575	8.0	7.3	.935
9.0	7.5	.600	9.0	7.4	.910
10.0	7.6	.670	10.0	7.5	.870
10.5	8.2	.719	10.5	7.6	.960
10.8	8.8	.615	10.8	8.0	.952
11.0	9.1	.350	11.0	8.8	.400
11.6	9.5	.150	11.6	9.5	.150
12.0	10.0	.050			
	(2 weeks)			(5 weeks)	
5.0	4.1	.980	5.0	4.0	1.150
6.0	5.6	.830	6.0	5.5	1.049
7.0	6.8	.989	7.0	6.7	1.020
8.0	7.3	.990	8.0	7.2	.983
9.0	7.5	.870	9.0	7.5	.900
10.0	7.7	.973	10.0	7.7	.973
10.5	8.0	.995	10.5	8.0	.995
10.8	8.7	.945	10.8	8.7	.945
11.0	8.9	.600	11.0	8.9	.600
11.6	9.5	.150	11.6	9.5	.150



Tanning in Borate Buffers.

The next possibility to be considered was the possible effect of the nature of the buffer system on the results obtained. In order to investigate this, it was desired to study the weight increase of collagen in quinone solutions buffered by salts other than phosphates, and for this purpose a borate system was shown. The solutions were made by using appropriate mixtures of boric acid and sodium tetraborate. Due to the limited solubility it was not possible to use 0.1M solutions in this case, and saturated solutions were used instead. Otherwise, the procedure was identical with that already described.

The results of their experiments are presented in Table III and Figure 6. The great influence of the nature of the system on the results obtained is immediately apparent from these curves. The alkaline maximum and the minimum point has disappeared, and instead, there is a single maximum which gradually shifts toward the acid range with increasing time. It is clear that an entirely different set of phenomena occurs in the borate buffered systems, as compared to those buffered with phosphate.

General Interpretation of the Weight Increase Curves.

Consideration of the weight increase curves for the phosphate buffered systems, and comparison of these with those obtained in the presence of borate, indicate that the

phenomena observed related not merely to the fixation by collagen of quinone alone, but rather to that of quinone plus an additional substance or substances. These additional substances must be derived from the quinone itself, and processes which immediately suggest themselves as means by which this might occur are oxidation and polymerization. The rapid reaction of quinone in even mildly alkaline solution is well known, and the work of Diels and Kassebart (8), and of Diels and Preiss (9), attests the fact that quinone is capable of polymerization. X-ray studies (17) of quinone tannage have already indicated the probability that a polymer of quinone is involved.

Inspection of the curves of Figure 4 and 5 suggests that these curves, obtained in phosphate buffered systems, are composites representing the fixation by the collagen of two different substances. As a preliminary working hypothesis, we may interpret these curves, as follows:

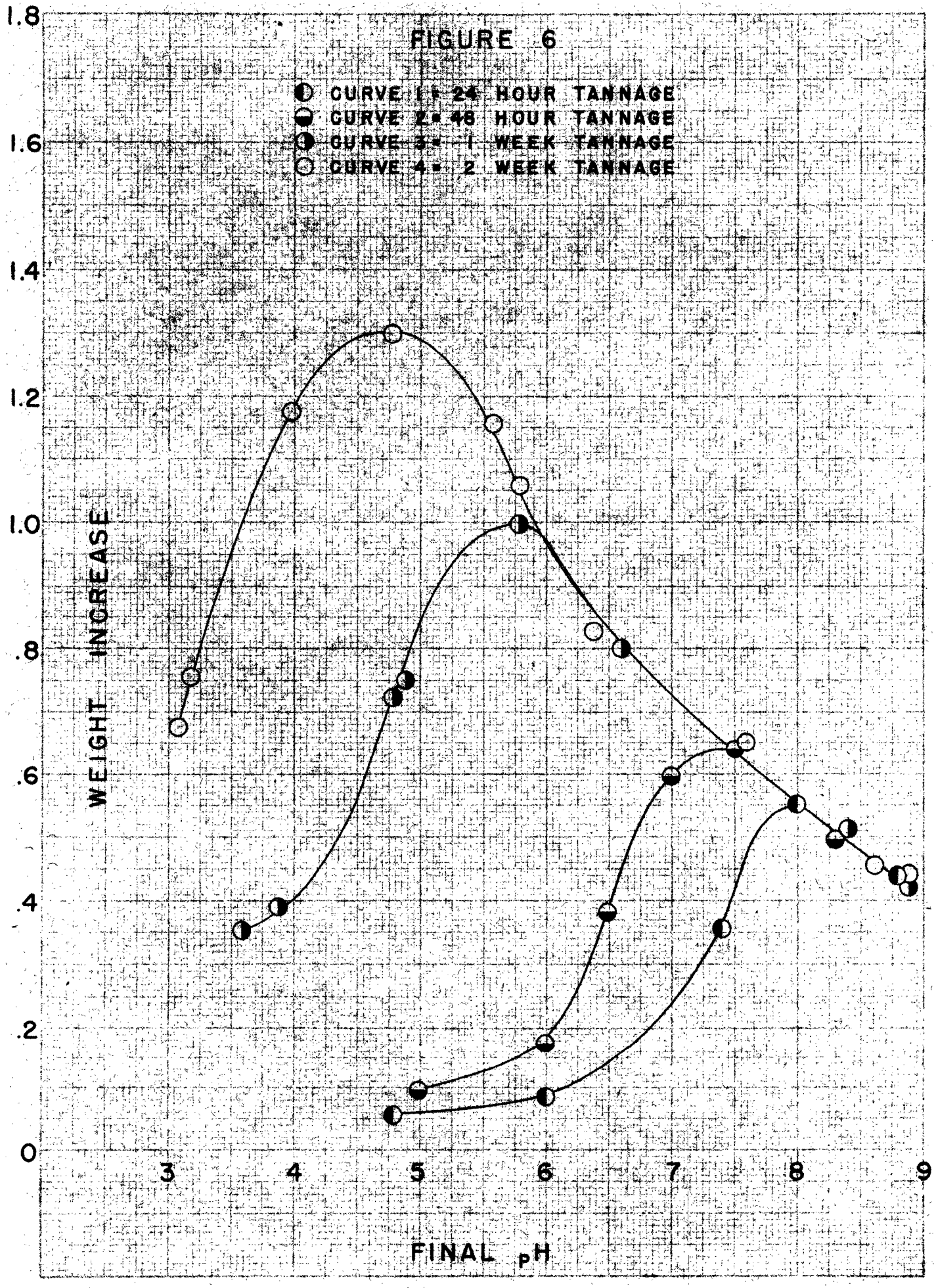
Considering, first, the three lower curves representing the shorter time periods, it may be assumed that the branches of the curves extending from the most acid value up to the maximum at pH 7.0-7.2 represent the combination of monomeric quinone with the collagen. This combination is apparently favored by the increasing alkaline reaction. As the reaction becomes still more alkaline,

TABLE III

Weight Increase of Collagen Powder
in Borate Buffered Quinone Solution

(2.000 gms. Collagen in 200 ml. Solution)

Initial pH	Final pH	Weight Increase	Initial pH	Final pH	Weight Increase
	(24 hours)			(48 hours)	
5.8	4.8	.056	6.5	5.0	.097
7.5	6.0	.085	7.5	6.0	.175
8.0	7.4	.355	8.0	6.5	.382
8.5	8.0	.552	8.5	7.0	.598
8.75	8.4	.515	8.75	8.4	.515
9.0	8.8	.440	9.0	8.8	.485
	(1 week)			(2 weeks)	
5.8	3.6	.335	5.8	3.1	.675
6.5	3.9	.390	6.5	3.2	.755
7.5	4.8	.725	7.5	4.0	1.175
7.75	4.9	.750	7.75	4.8	1.298
8.0	5.8	.998	8.0	5.6	1.155
8.5	6.6	.800	8.5	5.8	1.055
9.0	7.6	.650	9.0	6.4	.825
10.7	8.9	.442	9.75	7.5	.637
			10.7	8.3	.495
			10.9	8.6	.458
			11.3	8.9	.420



however, the polymerization of the quinone is increased, to such a point that it predominates over the combination reaction, and the fixation of the quinone drops off. At a still higher pH value (7.5) the effect of the quinone polymer on the collagen is becoming noticeable, and this effect predominates until about pH 8 is reached. Above this value the oxidation is stepped up so tremendously that the fixation falls off sharply. This explanation accounts very satisfactorily for the two maxima and the minimum point at pH 7.5. It is necessary to assume that the polymer is formed over the whole pH range, being formed more rapidly, however, in alkaline solution, but that it only begins to combine appreciably with the collagen above pH 7.5. The polymer is insoluble in acid, but soluble in alkali, and the marked increase in weight in the acid range over long periods of time is to be attributed to the slow formation and precipitation of the insoluble polymer in that range, in which form it is not combined with the collagen, but merely mechanically held among the fibers. These assumptions as to the behavior of the quinone polymer are in agreement with results obtained in an investigation of the nature of the polymerization, as reported subsequently in this paper. Microscopic examination also confirms the above interpretation, as many dark particles of deposited insoluble material can be seen among the fibers

tanned below pH 7.5, while above this point they are very much less in evidence.

Effect of Alkaline Buffered Wash Water on Weight Increase Curves.

Due to the solubility characteristics of the polymers of quinone, it was deemed advisable to study the effects of alkaline buffered wash water on the weight increase curves. There is an inherent source of error in this experiment because of the fact that the alkalinity of the wash water causes a quick take-up of "tannin," especially in the acid region. It is therefore to be expected that the weight increase curves represented in Figure 7 do not give the absolute values for weight increase at the various indicated pH values. These curves merely attempt to show that the usual type of washing procedure does not remove all, or nearly all, of the combined "tannin." This is best illustrated in Figure 8, where the extreme difference between collagen washed with distilled water and collagen washed with alkaline buffered distilled water are shown.

The Effects of Pre-Aging and of Accelerated Oxidation.

If it is true that in these solutions we are dealing with mixtures of quinone and substances derived from quinone by oxidation and polymerization, processes which require time, then it is logical to suppose that if

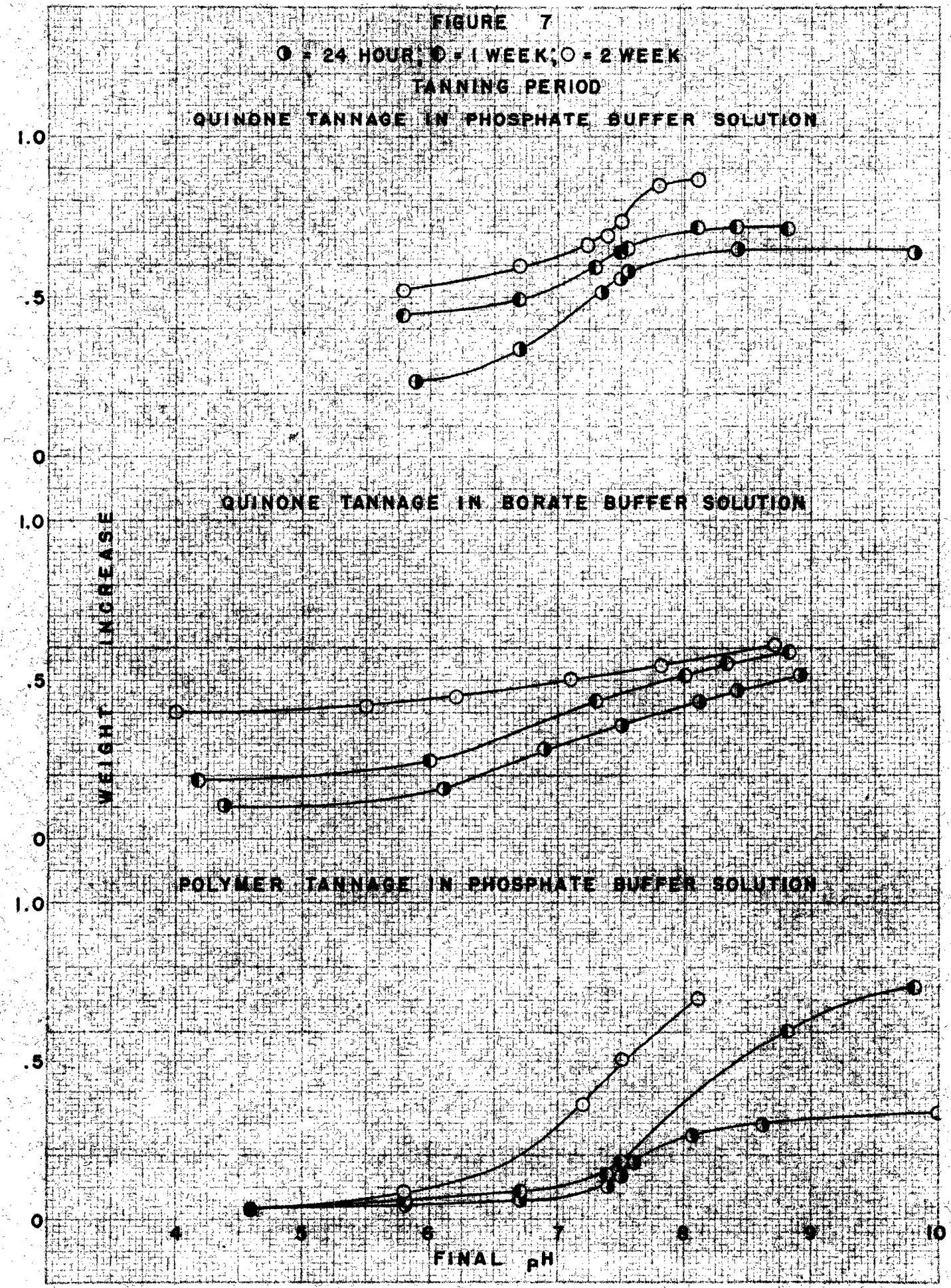
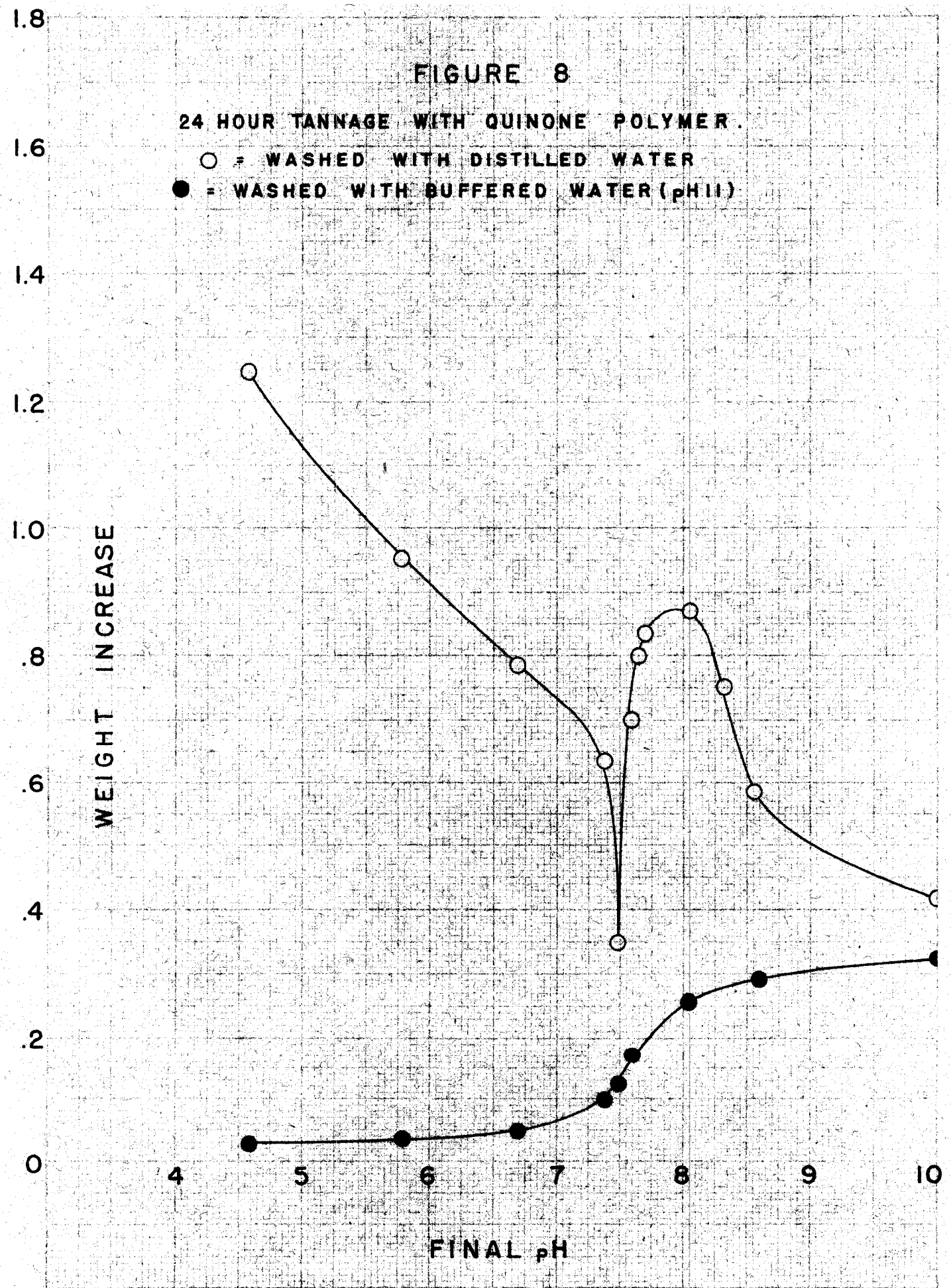


FIGURE 8

24 HOUR TANNAGE WITH QUINONE POLYMER.

○ = WASHED WITH DISTILLED WATER

● = WASHED WITH BUFFERED WATER (pH 11)



the solutions were aged before being allowed to react with the collagen, some distortion or change in the curves should result. Following this line of reasoning, an experiment was carried out in which the solutions were prepared exactly as before, using the phosphate buffer, and then allowed to stand at room temperature for two weeks before the addition of the collagen. Weight increases of the collagen were determined by the usual procedure at the end of a 24 hour tanning period. The results are presented in Table IV and Figure 9.

It is seen from Figure 9 that aging the solutions before tanning has resulted in the shifting of both maxima and the minimum point to lower pH values, and that the alkaline maximum has been markedly diminished. It is also noted that the weight increase in the acid range (below pH 6.5) has been very considerably increased. In other words, the concentration of the material causing the slow weight increase in the most acid region of the normal curves has been increased by aging the solutions before tanning, while the concentration of the substance causing the weight increase above pH 7.5 has been decreased. This is in line with the interpretation given above.

Since the decrease in fixation above pH 7.5 is probably due to the destruction of quinone or of the quinone polymer by oxidation, it should be possible to eliminate

TABLE IV

Weight Increase of 2 Grams Collagen Powder
Tanned for 24 Hours in 200 ml.
Phosphate Buffered Quinone Solution

(Solution Aged 2 Weeks Before Use)

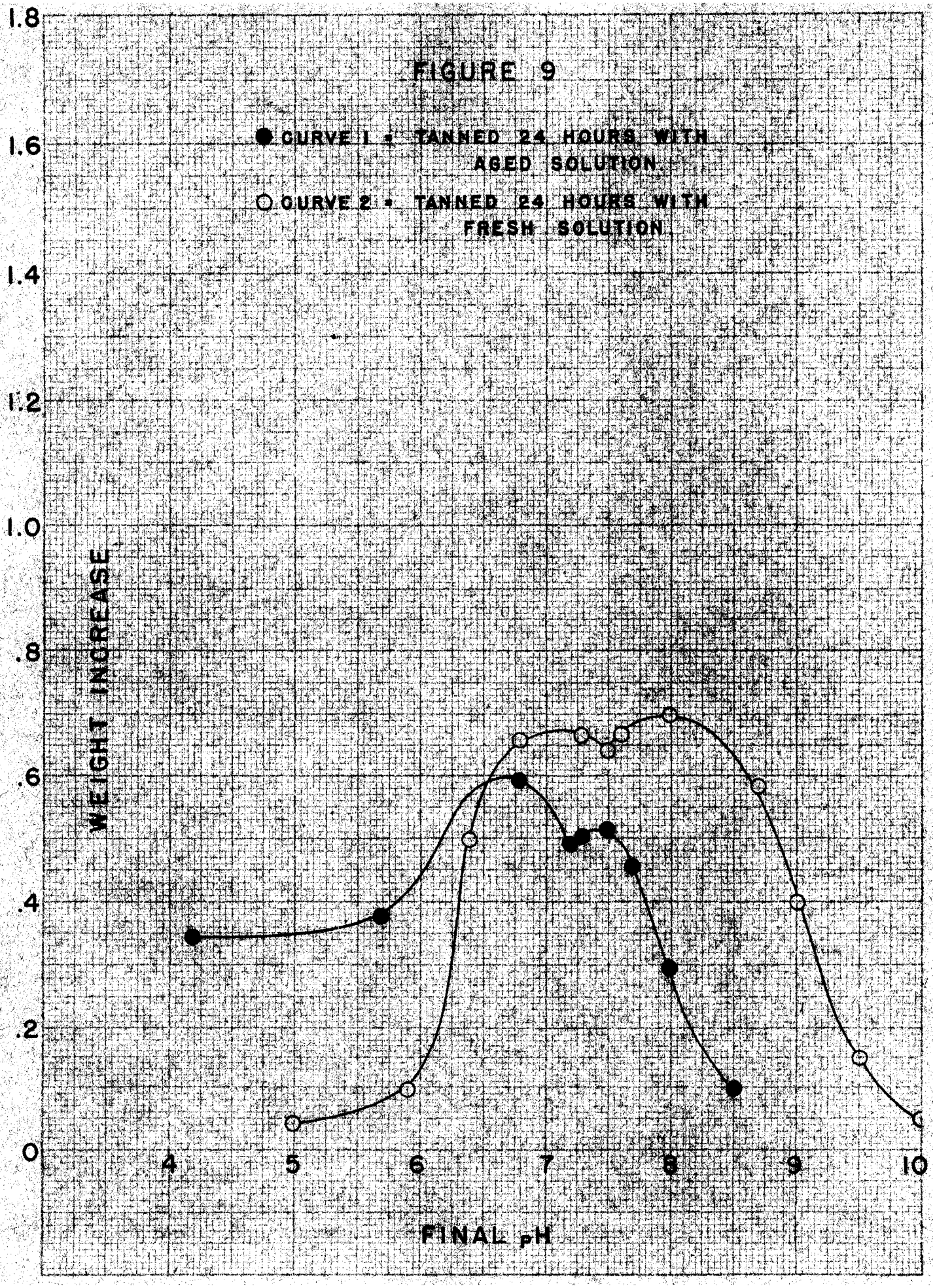
Initial pH	Final pH	Weight Increase
5.0	4.2	.347
6.0	5.7	.379
7.0	6.8	.594
8.0	7.2	.489
9.0	7.3	.502
10.0	7.5	.515
10.8	7.7	.457
11.6	8.0	.298
12.0	8.5	.100

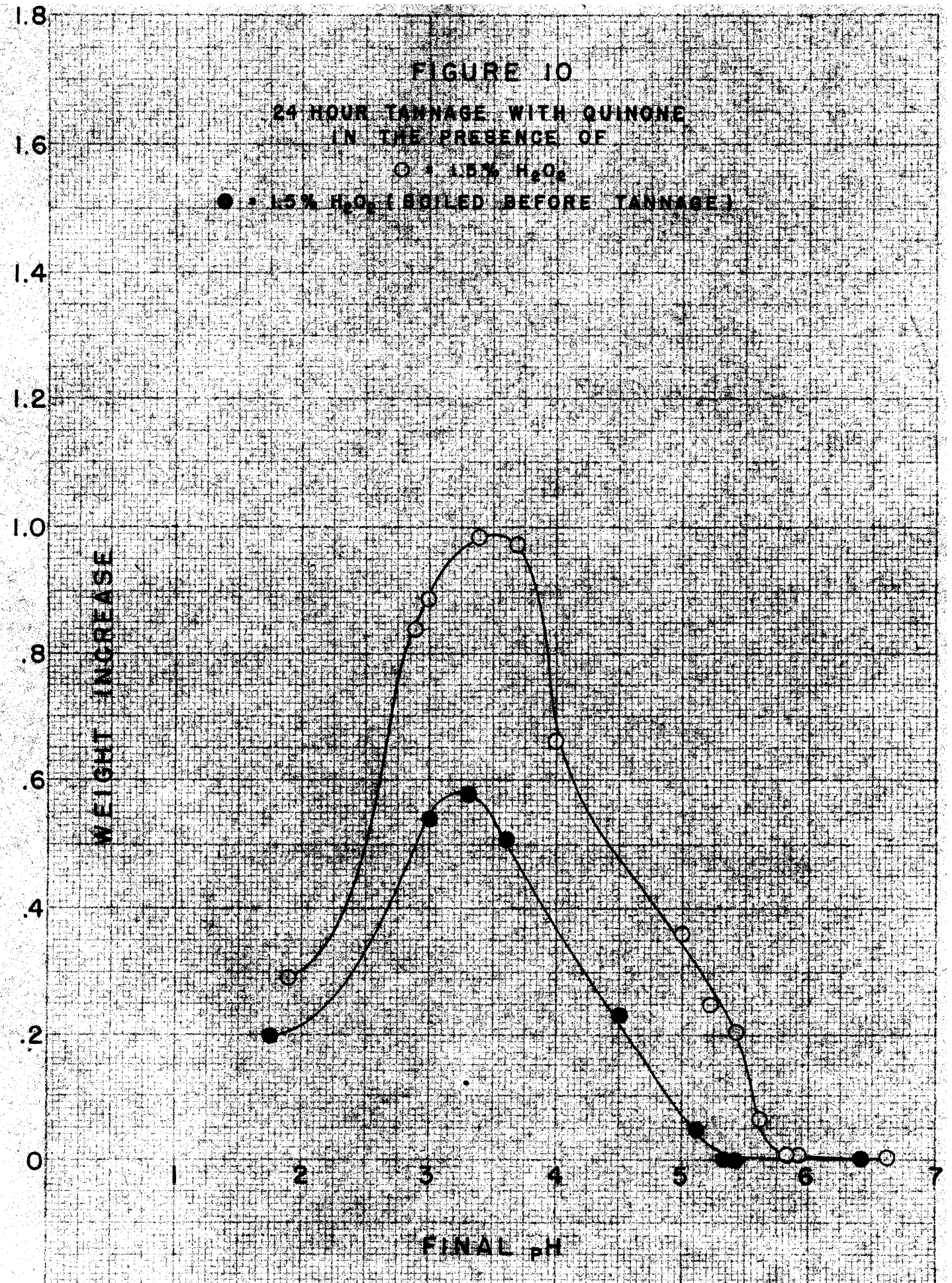
TABLE V

Weight Increase of Collagen Powder
in Phosphate Buffered Quinone Solution
Containing 1.5% H_2O_2

(2.000 gms. Collagen in 200 ml. Solution)

Initial pH	Final pH	Weight Increase
2.0	1.9	.292
3.9	2.9	.840
5.0	3.0	.885
5.5	3.4	.984
6.0	3.7	.972
6.3	4.0	.660
6.7	5.0	.364
7.0	5.2	.250
7.2	5.4	.204
8.0	5.6	.064
8.7	5.8	.004
10.0	5.9	.010
11.0	6.6	.005





this portion of the curve entirely by resorting to some sort of accelerated oxidation. To test this possibility an experiment was carried out in which the solutions were prepared in the usual way, using the phosphate buffer, and 1.5 per cent hydrogen peroxide was added. Table V and Curve 1 of Figure 10 give the results obtained on tanning for 24 hours in these solutions. It is seen that the accelerated oxidation has resulted in the complete disappearance of the alkaline maximum and, necessarily, of the minimum point. The acid maximum has been shifted to a still lower pH range, and also increased very considerably. The latter fact leads to the conclusion that at least part of the weight increase in the most acid region is due to the deposition of fixation of oxidation products. Curve 2 of Figure 10 illustrates the effect of a still more drastic oxidation, everything being identical with the previous procedure, except that the tanning solutions were heated to boiling and cooled before the addition of collagen sample.

The great similarity of the curves of Figure 10 with the curve in Figure 6, representing the results obtained on two week tannage in borate buffer systems will be noted. If the previous interpretation is correct, there will be a great difference in the oxidation of quinone in a borate system as compared to that in a phosphate

system, since the results in the borate system appear to correspond to those obtained in phosphate under conditions of greatly accelerated oxidation. In order to obtain a relative measure of the extent of oxidation of quinone in the two systems, quinone solutions were made up as before in phosphate and in borate buffer covering the pH scale from 2 to 10. These solutions were allowed to stand at room temperature for 24 hours, and the extent of oxidation in each solution then estimated by a modified "oxygen required" determination (20). This determination is a measure of the oxygen required to complete the oxidation, and is carried out by boiling an appropriate aliquot of the sample solution with dilute sulfuric acid and a known excess of standard potassium permanganate solution for 10 minutes, adding a known excess of standard oxalic acid solution, and back titrating with the standard permanganate solution until a pink coloration persisting for 30 seconds is reached. The amount of standard permanganate, thus determined, required to complete the oxidation of the quinone, subtracted from the amount required to completely oxidize a freshly prepared solution containing the same amount of quinone, is a measure of the air oxidation of the quinone during the 24 hour period.

The results are given in Table VI and Figure 11. It is apparent that a great difference in the oxida-

TABLE VI

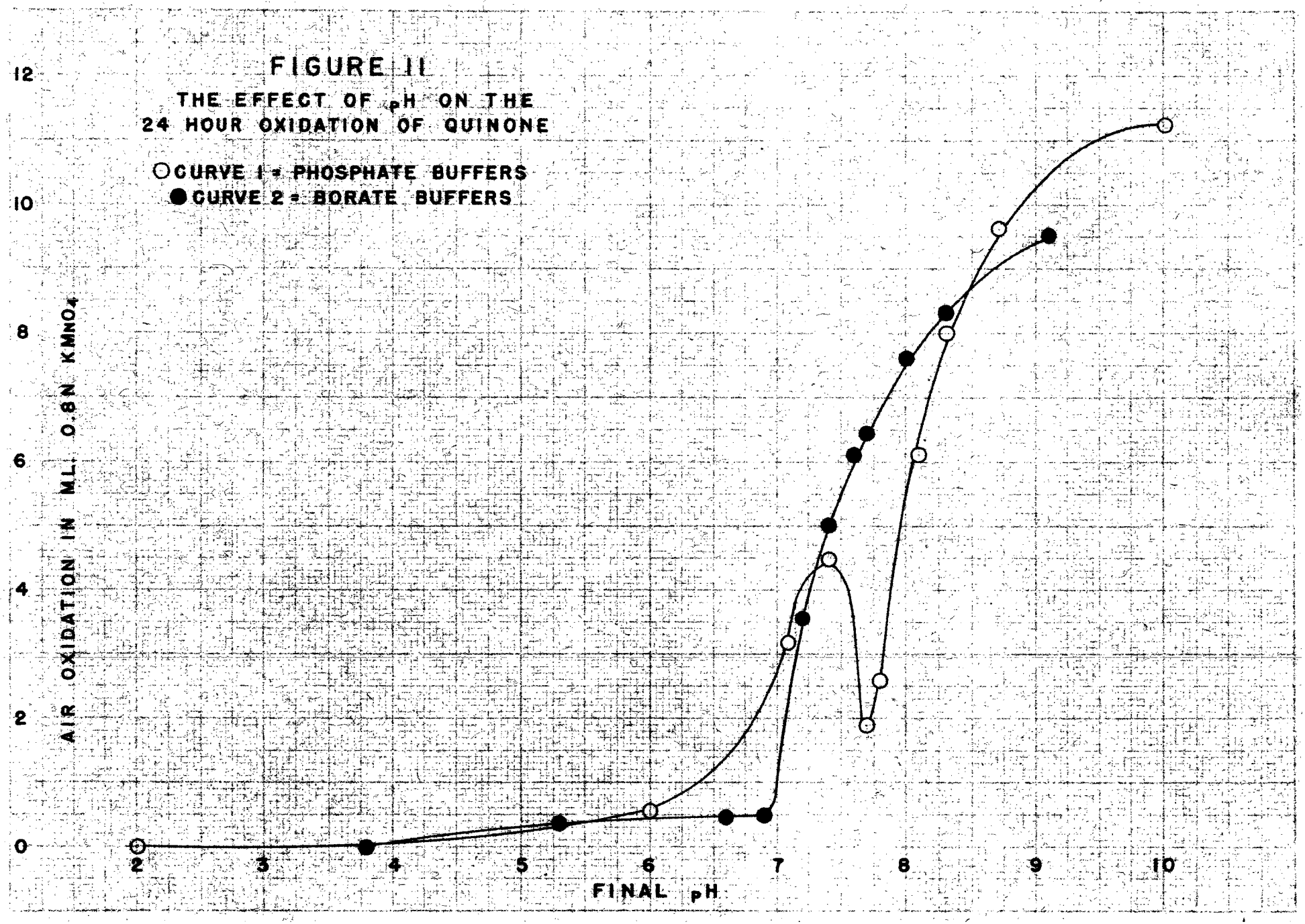
Effect of pH on Oxidation of Quinone
During 24 Hour Oxidation Period

(0.1 Gram Quinone in 200 ml.)

Phosphate Buffer			Borate Buffer		
Initial pH	Final pH	ml. 0.8N $KMnO_4$	Initial pH	Final pH	ml. 0.8N $KMnO_4$
2.0	2.0	0.0	3.7	3.7	0.0
6.0	6.0	0.6	5.3	5.3	0.4
7.2	7.1	3.2	6.7	6.6	0.5
7.7	7.4	4.5	7.0	6.9	0.5
8.0	7.7	1.9	7.5	7.2	3.6
8.1	7.8	2.6	7.6	7.4	5.0
8.7	8.1	6.1	8.0	7.6	6.1
10.0	8.3	8.0	8.1	7.7	6.4
10.2	8.7	9.6	8.2	8.0	7.6
10.7	10.0	11.2	8.5	8.3	8.3
			9.3	9.1	9.5

FIGURE II

THE EFFECT OF pH ON THE 24 HOUR OXIDATION OF QUINONE



tion of quinone in the two systems actually exists. In the borate system little oxidation occurs until about pH 7, where there is a sharp and regular increase along a smooth curve. In the case of the phosphate appreciable oxidation commences at about pH 6, rises to a maximum at pH 7.4, decreases to a minimum at pH 7.7, and then increases regularly. This is in good agreement with the interpretation previously given of the phenomena occurring in the phosphate systems. The inflection in the curve may be ascribed to the formation of a polymer which is more resistant to oxidation than quinone itself, and the oxidation of this polymer as a result of the increasingly alkaline reaction. In the borate system this polymer is not formed, and the curve may represent the oxidation of quinone itself.

The Formation of Quinone Polymers.

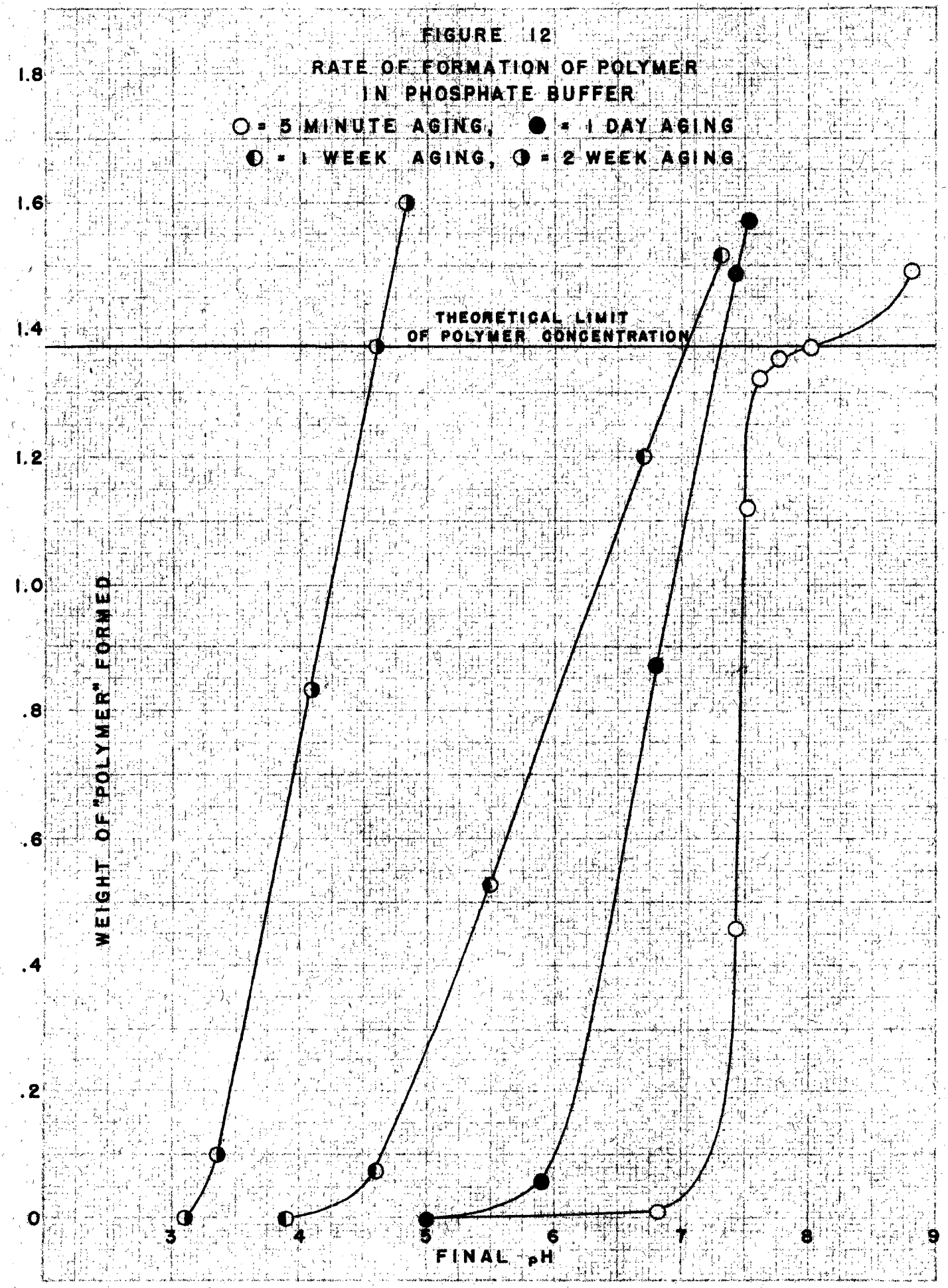
Earlier in this paper it was theorized that the minimum point in the weight increase curve in quinone - phosphate buffer tannage was due to the formation of a polymer of quinone. In an effort to obtain specific data concerning the formation of this polymer, a colorimetric quantitative estimation procedure was developed. It was found that by the use of a Wratten E. filter No. 22 (yellow), all concentrations of quinone produced a zero

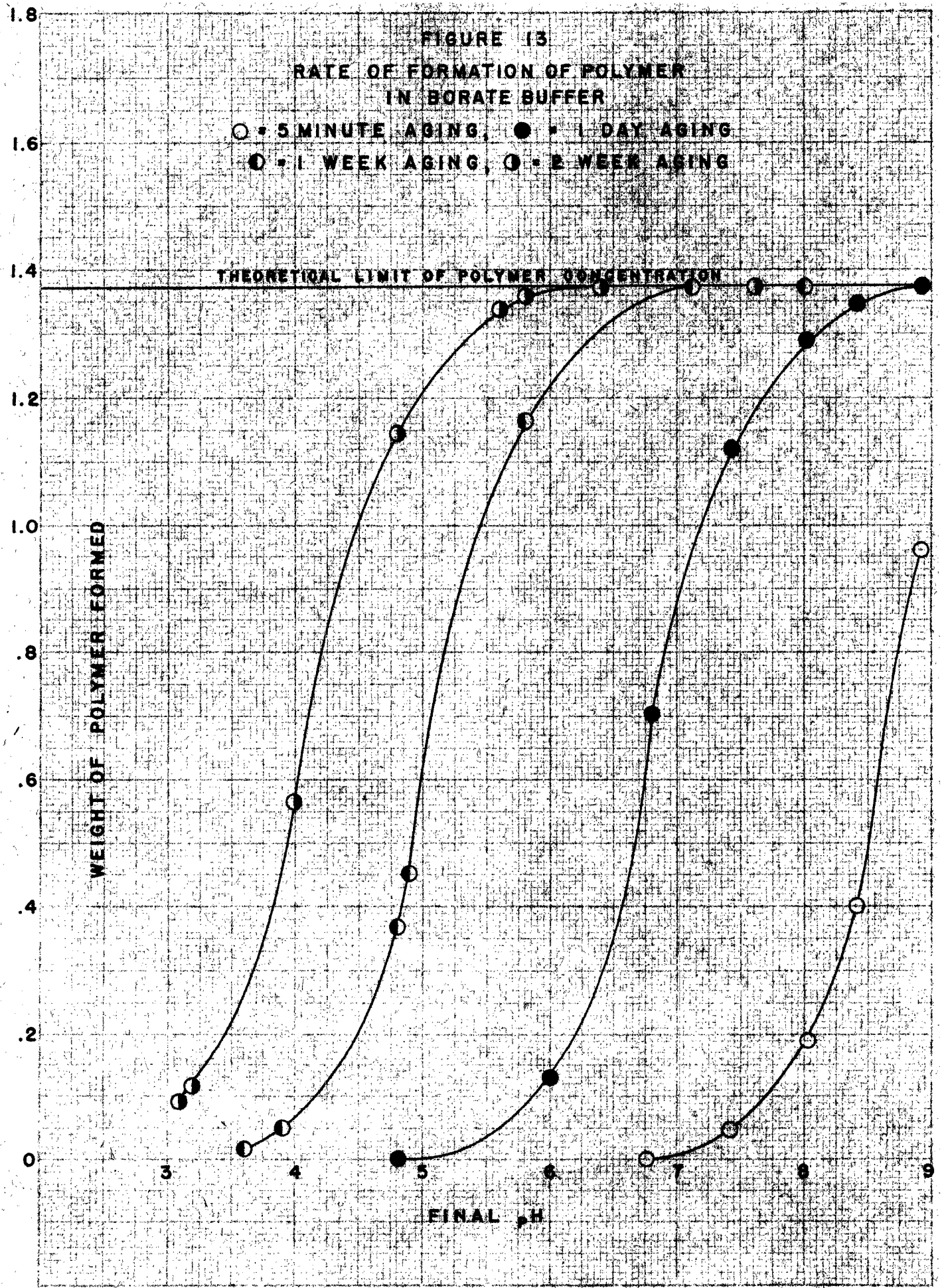
reading on the colorimeter, while the intense color of the polymer could be determined with a considerable degree of accuracy by referring the colorimeter values to a standard curve prepared with repurified polymer.

Figure 12 described the polymer formation over a range of time periods. It may be seen in the five-minute aging period, by the steep increase in formation at about pH 7.5, that this is very likely the reason for the minimum point in the weight increase tannage curves. The curves produced during longer time periods substantiate the proposed explanation for the rise with time of the weight increase curves in the acid region.

The same type of quantitative procedure was established for the determination of the borate polymer. The curves represented in Figure 13 show the completely different method of formation of the polymer in borate buffer solutions. They also indicate an explanation for the weight increase curves in quinone-borate tannages.

In the molecular weight determination of the polymer of quinone formed in aqueous alkaline solution, the modified Barger method was found to be at best only a rough approximation of the true weight. Capillary tubes were filled, according to specified techniques, with a range of molarity between 0.000195M and 0.01250M. Distillation occurred in the direction of the urea standard down





to a concentration of 0.00078M, but there was no noticeable distillation in either direction in the tubes of lesser concentration. This may have been due to the fact that the difference in osmotic pressure between the standard and polymer was too small to yield practical data under conditions of such dilution. The important information derived from this data, however, is that the molecular weight of the polymer must be greater than 2500, as calculated from the formula:

$$\frac{\text{grams/liter of sample}}{\text{molar concentration of sample}} = \text{molecular weight of sample}$$

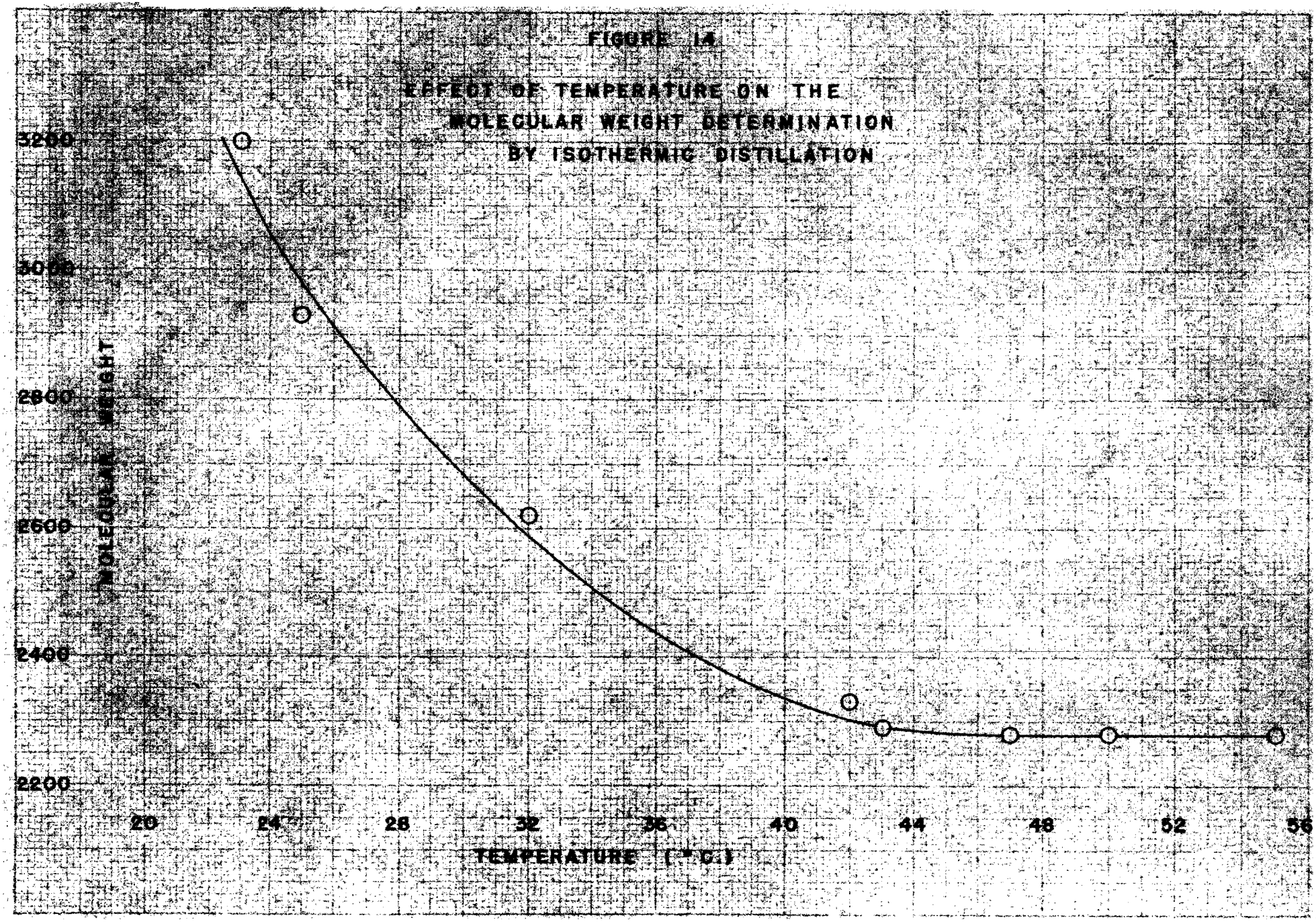
The Signer method of isothermic distillation for the determination of molecular weight has proven to be much superior for accurate measurements. Azobenzene, the standard chosen for this experiment, was deliberately made up to a lower molarity than that suspected for the polymeric solution, so that any distillation would take place in the direction of the polymer and preclude the possibility of its concentration and precipitation from solution. The progress of the distillation of the acetone solvent was checked daily by measuring the volume of solution in each arm of the apparatus until no more transfer occurred. At this time the volumes and concentrations of the sample and standard solutions, together with the molecular weight of the standard were inserted into the

equation representing Raoult's Law. The interesting result of this experiment was that the molecular weight tended to decrease with increasing temperature until a constant value was reached at about 43° Centigrade. Figure 14 graphically illustrates the course of this molecular weight depression. The minimum molecular weight calculated from this data is 2280. It may be reasoned that the lowering of the molecular weight of the polymer with increased temperature is an indication of its dissociation, and that at low temperature the molecular weight may reach a maximum by association. However, the apparent weight may be expected to increase as the higher polymers are formed and become insoluble in the solution.

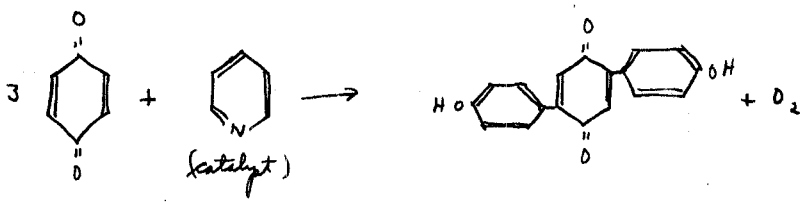
During the process of investigating the characteristics of the quinone polymer, it was noted that the compound possessed very noticeable acidic properties. Further work revealed that it was possible to titrate the acidity of the polymer with a fair degree of accuracy, although the neutralization equivalent did not have any particular significance since it is probably a very low submultiple of the true weight. But, whatever the true molecular weight of the polymer may be, since it is of a fairly high order of magnitude, an interesting problem of linkage and structure awaits to be developed.

FIGURE 14

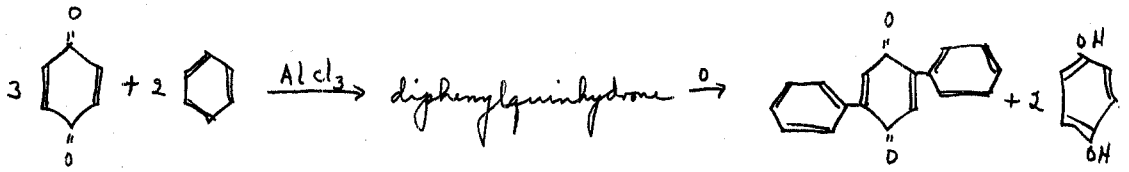
EFFECT OF TEMPERATURE ON THE MOLECULAR WEIGHT DETERMINATION BY ISOTHERMIC DISTILLATION



According to Diels and Kassebart (8), quinone forms a trimer in the presence of pyridine:



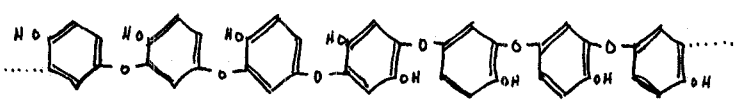
This reaction displays the general coupling position tendencies of quinone which are of utmost importance in developing a provisional structure for the quinone polymer. It has been found, too, that quinone and benzene in anhydrous aluminum chloride suspension utilizes the same coupling positions (25), although the conditions of reaction are very dissimilar:



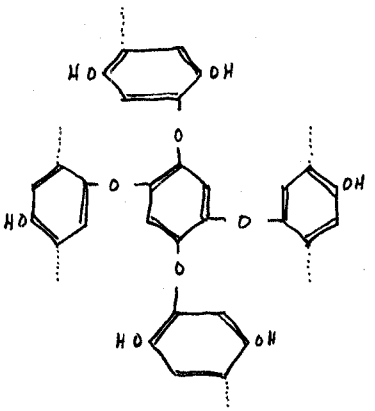
This would seem to indicate that, with possible exceptions, the usual linkage positions over a considerable range of conditions would remain the 2,5- type.

With these facts in mind it is possible to construct a feasible form (or forms) of structure. For example:

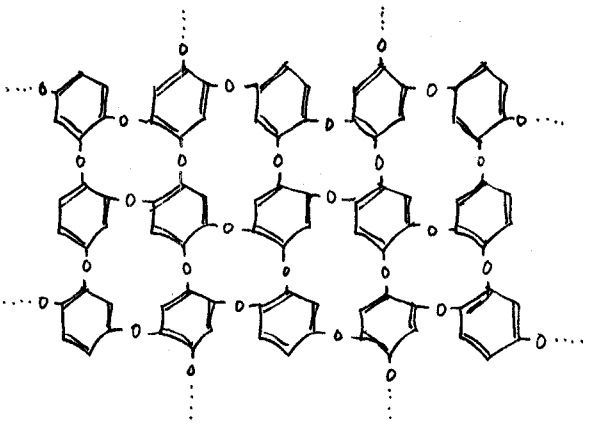
(I)



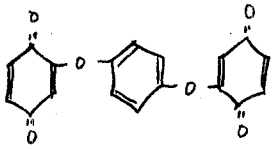
(II)



(III)



Woker and Antener (40) have reported a linkage,



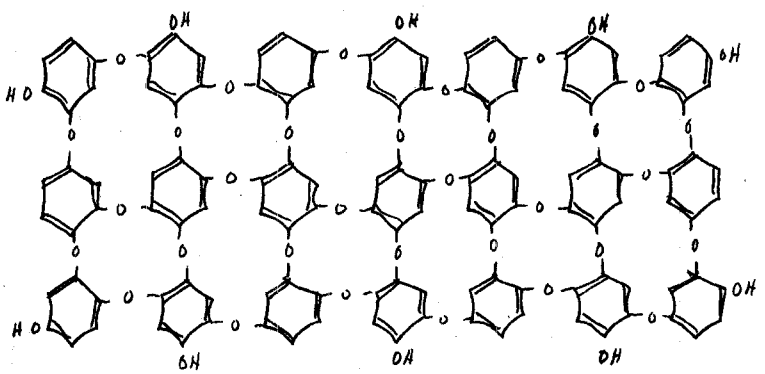
which would seem to indicate that III is perhaps the most plausible structure. In any case, it becomes evident from a study of the above structure forms that a determination of the hydroxyl content of the polymer might be of great value in choosing the most plausible type.

Several estimations, direct and indirect, of the hydroxyl content of the polymer were conducted. In

the first investigation, the determination of the hydroxyl number by the Roberts-Schuette method, it was found that the hydroxyl groups composed roughly 4.5% of the total polymer weight. This quite definitely established the fact that I and II were not possible because their minimum percentage of hydroxyl groups must necessarily be greater than 18%, from structural considerations. It is still possible, however, that III may be the correct concept of structure, since this type of coupling may occur in all directions with the only limitations being steric hindrance effects or removal from reaction by insolubilization.

The use of methylation as an indirect means of determining hydroxyl content has led to the further discovery that all of the hydroxyl groups are fairly uniformly acidic in nature. The average percentage of hydroxyl groups by the diazomethane and the dimethyl sulfate procedures was found to be 6%, a slightly higher value than that found by the Roberts-Schuette method, but, nevertheless, it precludes the possibility of using I and II. It is very likely in this connection that the insolubility of the polymer in the Roberts-Schuette reaction mixture would account for the lower results; the methylation procedures appear to be superior in dealing with this polymer.

Now, knowing the approximate molecular weight and the average hydroxyl content of the compound, it should be possible to establish its provisional structure at a given temperature. The following structure has been proposed for the polymer found at a temperature of 50° Centigrade in acetone solution:

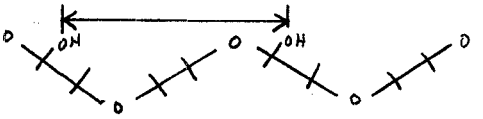


M W: 2246 ; % hydroxyl: 7.5

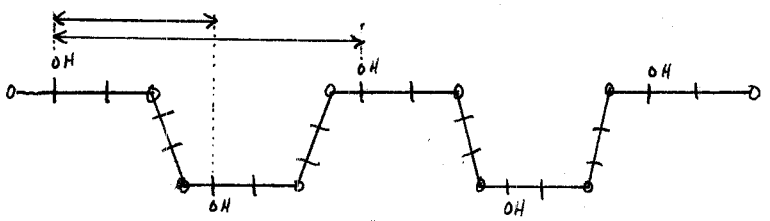
It should be borne in mind, however, that the above structure indicates only the approximate number of benzene nuclei, and that association may take place, utilizing all of the hydroxyl groups shown. It is quite probable in this connection that the polymer as it exists in water has a much higher molecular weight.

It may be said here that from the standpoint of atomic arrangement the polymer may be interpreted as roughly resembling a corrugated structure. By this is meant that the benzene nuclei are joined together in a

type of ether linkage, benzene ring to benzene ring through oxygen. The oxygen has been reported to have a valence angle of 128° in similar ether linkage, so for the purpose of showing a possible molecular structure, let us assume this angle to be the same in the polymer. Then, knowing the length of the benzene nuclei and the angle by which they are joined, it is possible to calculate the distance between crests or depressions along the polymer surface. In the case of the more extended form,



the distance between adjacent recurring units was calculated to be 9.6 Å and 10.3 Å. It is possible that another type of structure of the polymer may exist, in which adjacent hydroxyl groups, though not in the same plane, are about 8.6 Å apart, and adjacent recurring units are about 17.2 Å apart (three and six times the distance, respectively, between adjacent side chain units). Considering the long axis of the benzene nuclei, the equivalent distances are calculated to be 9.2 Å and 18.4 Å, respectively. This may point to a possible secondary valence bond formation between basic amino acids and the acidic hydroxyl groups of the polymer:

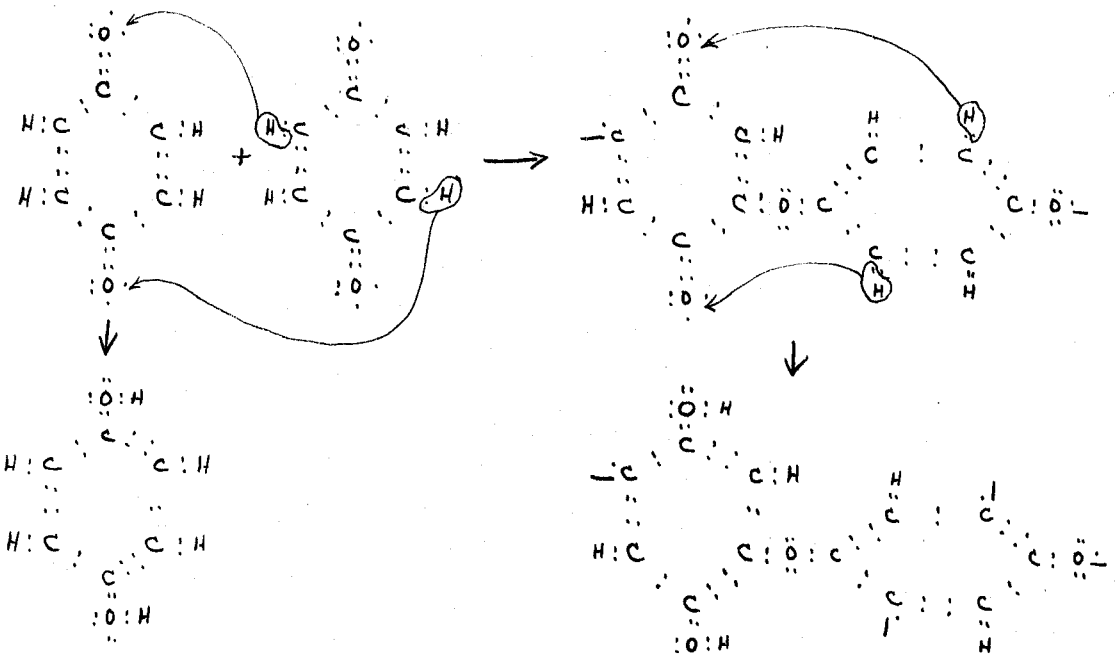


It is doubtful whether every possible side chain is linked with the polymer in any way, but they may be connected at random distances with sufficient force to prevent removal of the polymer by mild washing procedures. It is interesting to note that if these large polymeric molecules are taken up from a water "solution" by swelled collagen molecules and held firmly through the drying procedure, the collagen on drying to the normal size would be bent or distorted around these particles, thus accounting for the change in X-ray diagrams on quinone tannage.

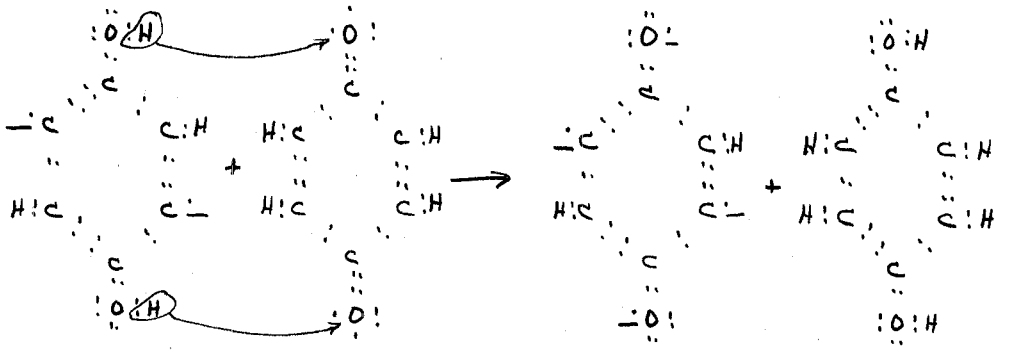
In order better to understand the mechanism of this polymer formation, a series of experiments were performed under varied conditions of formation. (1) It has been found that the type of buffer greatly influences either the type of polymer formed or its ability to tan. (2) Hydrogen peroxide and air oxidation tend to destroy the ability to tan in the alkaline range. However, the fact that polymer formation is not dependant upon oxygen was clearly shown in the following experiment. An H-tube was constructed so that an alkaline phosphate

buffer solution could be introduced into one arm and pure, dry quinone into the other. While a stream of pure nitrogen gas was passed through the tube each arm was heated so as to drive off residual dissolved oxygen in the solution and on the solid quinone. After this treatment the tube was sealed on both arms, being careful not to allow air to enter, and tipped so as to let the buffer solution flow over the quinone. Upon agitation the quinone dissolved and an immediate darkening of the solution was observed, the intense color of which betrayed the polymer formation. It is possible, however, that part of the quinone was used to replace the usual source of oxygen, and that the percentage yield was affected.

The following is a suggested electronic mechanism of polymerization:



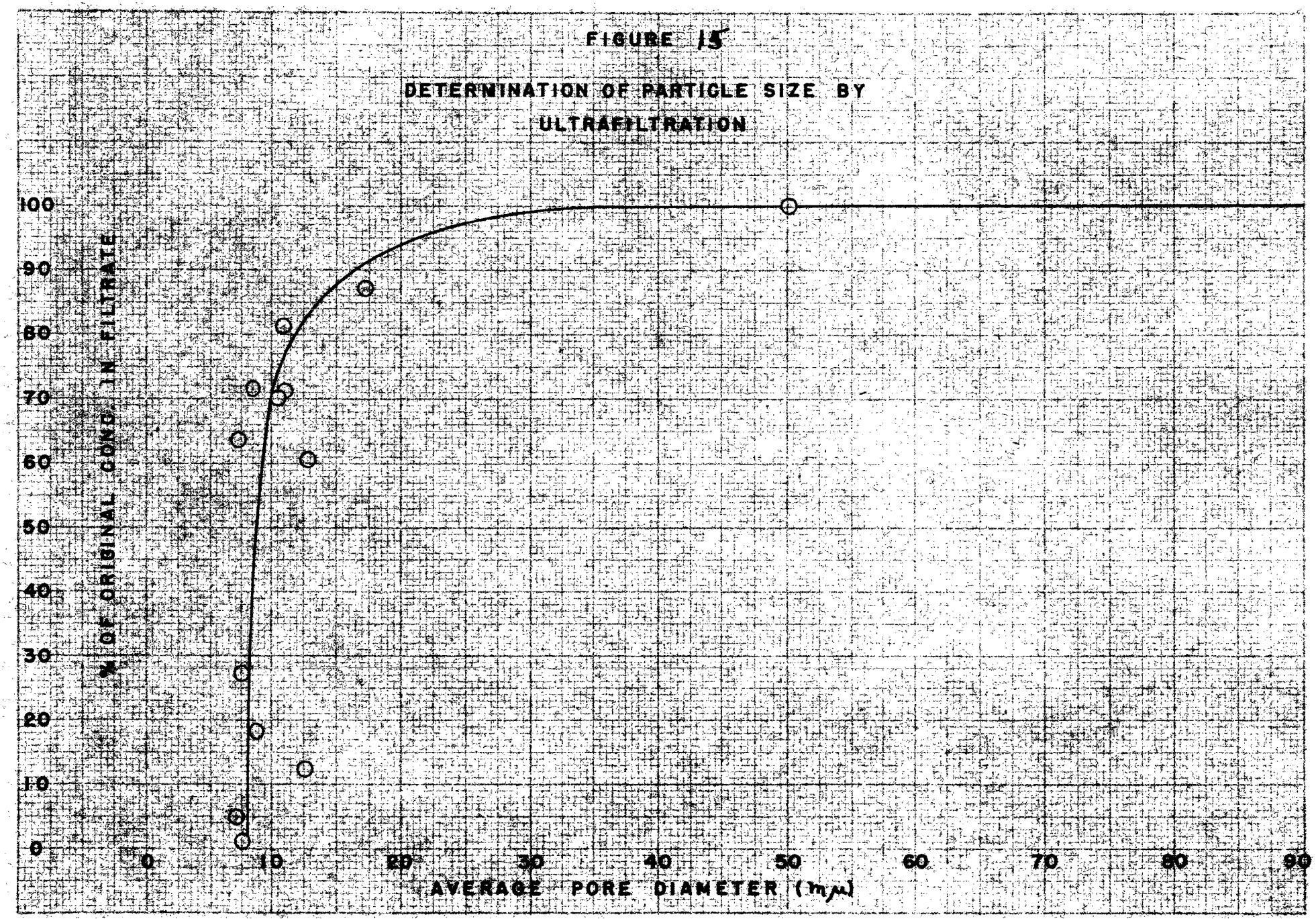
the probable catalytic action of quinone may be electronically illustrated:



Since the polymeric form of quinone has such a large molecular weight, with a limited number of solubilizing groups, its solubility is apparently very limited. In fact, it is not at all illogical to suppose that a true solution of the compound in water is difficult, if not impossible, to obtain. In order to check the possibility of the polymer being in a colloidal rather than a true solution, the method of ultrafiltration was employed. Solutions containing a known quantity of the polymer were made up at a pH value of 11.5, so as to assure the polymer being in solution as much as possible. Figure 15 illustrates the results obtained by plotting the average pore diameter of the filtration membranes against the percentage transmission of the polymer through the membrane. The particle size of the compound is clearly indicated to be

FIGURE 15

DETERMINATION OF PARTICLE SIZE BY ULTRAFILTRATION



less than 10 mu, and more probably in the neighborhood of 3-5 mu, which is below the microscopic visibility range. This fact may account for the apparent tanning action of the polymer as evidenced by the weight increase method of estimation; it may also account for its inability to penetrate hide substance, except with extreme difficulty.

Summary:

A quantitative study has been made of the combination of p-benzoquinone with collagen over a wide range of pH, and in consideration of a number of complicating factors in an attempt to check and elaborate upon the previous research done on this problem. In so doing, it has been found necessary not only to study the combination, but also to turn to the more fundamental exercise of studying the tanning agent itself.

The results may be interpreted as follows:

(1) The work of Thomas and Kelly has been checked and reported, using final pH values obtained through the use of the glass electrode.

(2) When quinone solutions (more particularly of high pH) are allowed to stand for a period of time, the quinone molecules interact to form a darkly colored, acidic substance of apparently high molecular weight. The polymer formed in phosphate buffered solutions is of a different nature than that formed in borate buffered solutions.

(3) The minimum point observed in the collagen weight increase curves in the phosphate buffered tanning solutions has been shown to be due to the character of the polymer formed rather than to any peculiarity of the collagen.

(4) Slow penetration of alkaline quinone tanning liquors has been attributed to the fact that polymer particles of sufficient size to affect a blocking phenomenon are produced.

(5) A type of linkage has been proposed for the polymer so as to account for the low hydroxyl content and relatively high molecular weight.

(6) Various conceptions of the chemical linkage of quinone to collagen have been reviewed.

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