resulting sirup was largely dissolved in 150 ml. of chloroform, cooled to room temperature and extracted, first with 300 ml. of 3 *N* ammonium hydroxide and then with water containing a little ammonia. Crude Ic was isolated and esterified as described for the corresponding operations in series b; the yield of crude IIc was 10.2 g. (23%). IIc crystallized from butanone in long faintly yellow needles, m. p. 160–160.5°, $\lambda_{max.}$ 264–266, 372–373 mμ.

Anal. Calcd. for $C_{19}H_{16}N_2O_5$: C, 64.77; H, 4.58; N, 7.95. Found: C, 64.81; H, 4.42; N, 8.31.

Ethyl 2-Phenyl-6-methoxy-8-aminocinchoninate (IIIc).[18] —The reduction of 4 g. of IIc (0.2 g. of platinum oxide and 200 ml. of methanol) required twenty minutes; IIc went into solution and near the end of the reduction, IIIc began to crystallize out. After redissolving and filtering from catalyst, 2.6 g. of large red needles was isolated by cooling the filtrate; dilution with water gave a second crop; the total yield was 3.55 g. (96%). Recrystallization from ethanol gave material having the same m. p. as the crude, 120–120.5°, $\lambda_{max.}$ 299–302, (*ca.* 355), 410–412 mμ.

Anal. Calcd. for $C_{19}H_{18}N_2O_3$: C, 70.79; H, 5.63; N, 8.69. Found: C, 70.84; H, 5.36; N, 8.76.

Summary

Several syntheses of 8-nitrocinchoninic acids have been investigated.

For preparation of 2-phenyl-8-nitrocinchoninic acid, a modified Doebner reaction has been developed; by use of this method 2-phenyl-6-methoxy-8-nitrocinchoninic acid has also been made available.

Catalytic reduction of 8-nitrocinchoninic esters (colorless) gave 8-aminocinchoninic esters (colored).

PASADENA, CALIFORNIA RECEIVED JUNE 25, 1946

[CONTRIBUTION FROM THE BIOCHEMICAL LABORATORY, STATE UNIVERSITY OF IOWA]

The Mechanism of the Antioxygenic Synergism of Quinones and Quinols with Phosphoric Acid and other Acids in Fat Systems[1]

BY VINCENT P. CALKINS[2,3]

The stabilizing action of traces of phosphoric acid, when added to certain fats, has long been known.[4,5] More recently it was demonstrated[6] that this action depended on the simultaneous presence of tocopherols or other phenolic inhibitors or their quinones and apparently consisted in a shift of the quinol ⇄ quinone equilibrium to the left, toward the reductant. The mechanism of this action is all the more intriguing because, although acids are known to favor this shift, the traces of phosphoric acid which impart increased stability are too minute to change the hydrogen ion concentration of the medium. The purpose of this investigation was to elucidate the mechanism by which a combination of quinones (or quinols) with traces of phosphoric acid achieves a much greater antioxygenic effect than is possessed by either alone, and to apply any resultant principles to other systems.

The fat substrate used was the ethyl esters of lard fatty acids, prepared in the usual manner and dehydrated by heating on a steam-bath under vacuum. When quinone was added its concentration was always 0.02%; in a third system the ethyl esters contained 85% orthophosphoric acid in two concentrations, 0.09 and 0.045%, and, finally, in a phosphoric acid–quinone–lard ethyl ester system, four different concentrations of phosphoric acid were used, namely, 0.09, 0.045, 0.009 and 0.0002%.

Quinone and phosphoric acid were incorporated into the fat system in the following manner. The solid ethyl esters, removed from cold storage, were liquefied by heating them gently under nitrogen on a steam-bath. A definite amount, weighed into a 400-ml. beaker, constituted the blank sample. In a larger amount of the esters sufficient quinone was dissolved to make a 0.02% solution. A portion of this, of the same weight as the blank, became the quinone–ester sample and like amounts were added to each of several 400-ml. beakers containing the desired quantity of phosphoric acid that had been weighed out on a cover slip and placed on the bottom of the beaker; ester solutions and esters were added carefully so as not to disturb the acid on the cover slip.

The beakers were covered with watch glasses and placed in an air oven at 60°; the air was stirred. At intervals quantitative determinations were made of the content of peroxides[7] and of quinols.[8] Since peroxides interfere with the Emmerie and Engel test, quinol determinations could not be made when appreciable amounts of peroxides were present.

Figure 1 presents a comparison of the appearance and disappearance of peroxides over a period of 140 days in samples containing quinone or phosphoric acid or neither. Both 0.09 and 0.045% of phosphoric acid showed a definite but

(1) The experimental data in this paper are taken from a dissertation submitted by Vincent P. Calkins to the Faculty of the Graduate College of the State University of Iowa in partial fulfilment of the requirments for the degree of Doctor of Philosophy, April, 1943. Presented in part before the Chicago Meeting of the American Oil Chemists Society, November 1, 1946.

(2) A part-time grant from Merck and Co. is gratefully acknowledged.

(3) Present address: Tennessee Eastman Corporation, Oak Ridge, Tennessee.

(4) E. W. Eckey, U. S. Patents, 1,982,907 (1934); 1,933,152 (1935).

(5) H. S. Olcott and H. A. Mattill, THIS JOURNAL, **58**, 2204 (1936).

(6) Calvin Golumbic, *Oil & Soap*, **19**, 181 (1942).

(7) R. B. French, H. S. Olcott and H. A. Mattill, *Ind. Eng. Chem.*, **27**, 724 (1935).

(8) A. Emmerie and C. Engel, *Rec. trav. Chim.*, **57**, 1351 (1938).

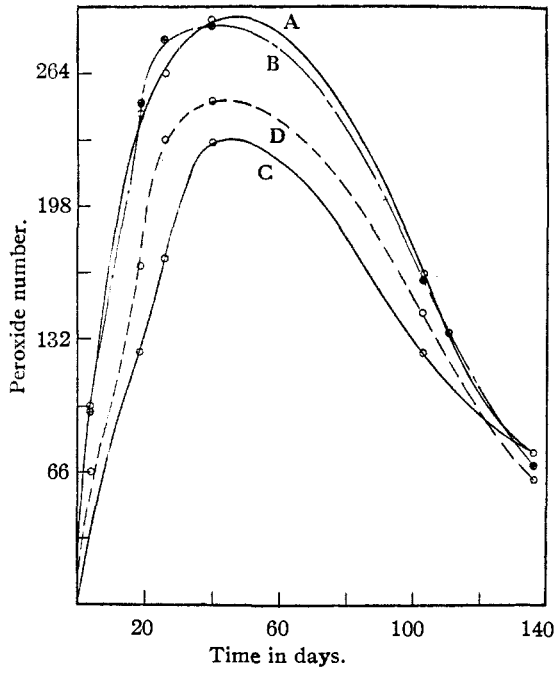


Fig. 1.—Peroxide formation and destruction during the autoxidation of lard ethyl esters: A, lard ethyl esters; B, 0.02% quinone-lard ethyl esters; C, 0.09% phosphoric acid-lard ethyl esters; D, 0.045% phosphoric acid-lard ethyl esters.

slight antioxidant effect when compared with the blank. A 0.02% solution of quinone in the esters showed no increased stabilization; the small amount of quinol that may have appeared by reduction of quinone[6] did not alter the results.

Figure 2 shows the variations in quinol content in a 0.09% phosphoric acid–quinone–ester system over a similar period. This substance is not actually a typical quinol, as will appear later.

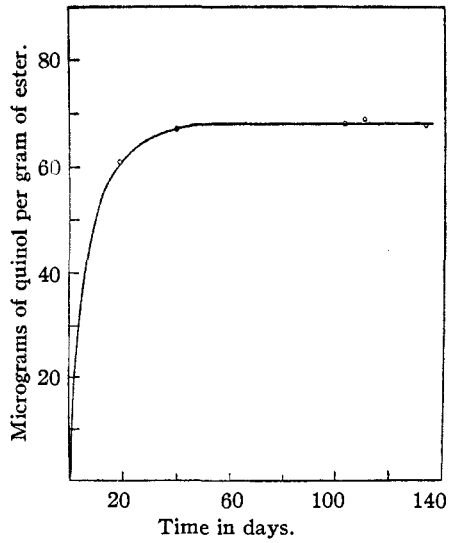


Fig. 2.—Quinol content of a quinone-phosphoric acid-fat ester system (quinone 0.02%, phosphoric acid 0.09%).

Amazingly, throughout this period of 140 days, the peroxide content was zero.

In Figure 3, curves A, B and C represent quinol formation in the first two weeks of the induction period in systems containing 0.02% quinone and 0.09, 0.045 and 0.009%, respectively, of phosphoric acid. The values in curve C may be slightly high due to the presence of a very small amount of peroxides.

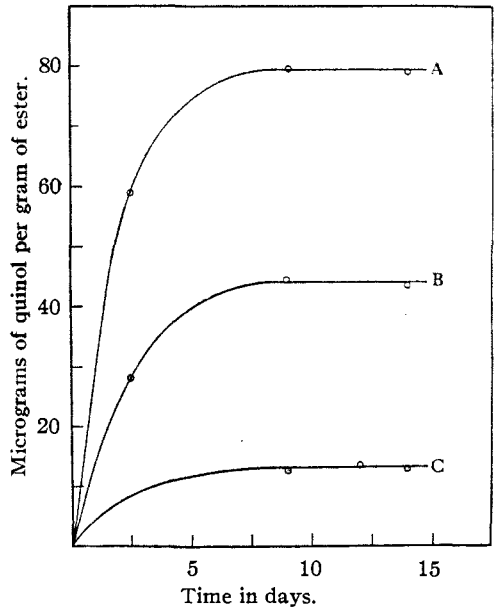


Fig. 3.—Quinol formation in a (0.02%) quinone-phosphoric acid-fat ester system containing (A) 0.09%, (B) 0.045% and (C) 0.009% of phosphoric acid.

Figure 4 shows peroxide formation with time in the quinone-ester substrates, containing (A)

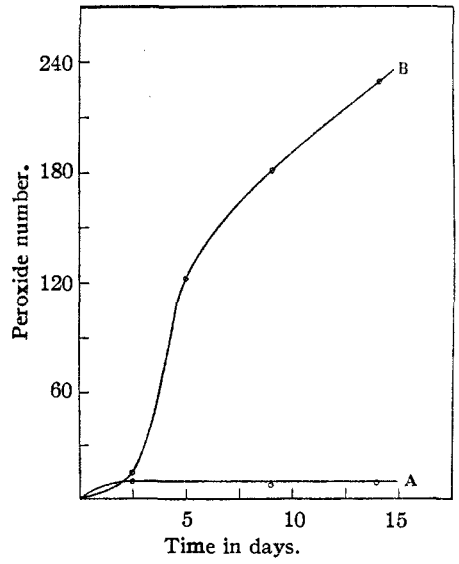


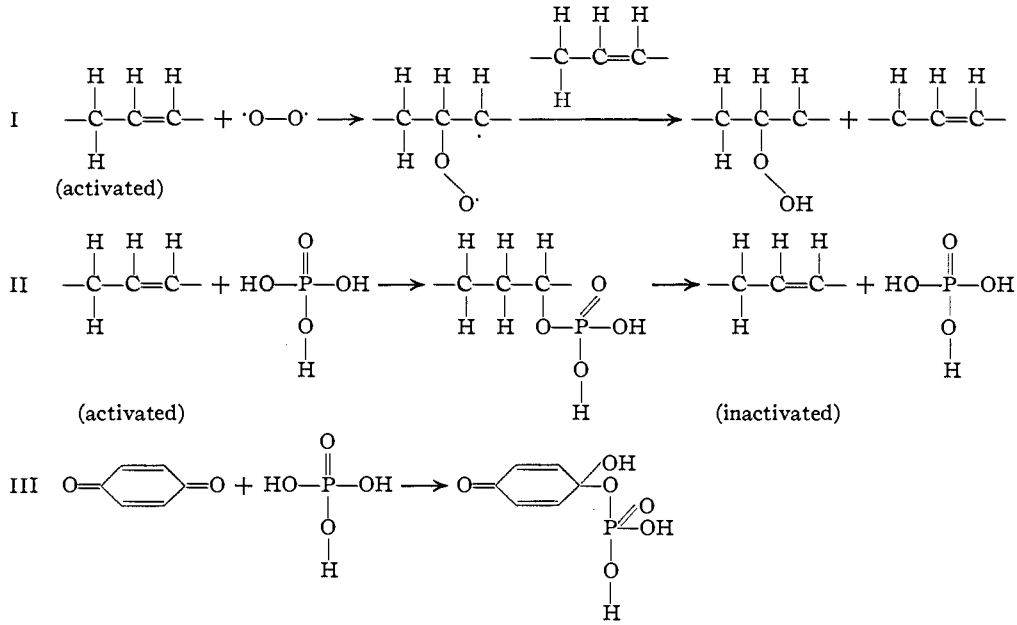
Fig. 4.—Peroxide formation in quinone-phosphoric acid-fat ester systems containing (A) 0.009% and (B) 0.0002% phosphoric acid.

0.009 and (B) 0.0002% of phosphoric acid, respectively. No peroxides were formed in quinone–ester systems containing 0.09 or 0.045% phosphoric acid.

Discussion

From visual observation, it appeared that only the 0.0002% phosphoric acid concentration in the lard esters constituted a homogeneous solution. The other three systems involving higher concentrations of phosphoric acid with or without quinone were heterogeneous systems; a small amount of phosphoric acid remained undissolved, separated by the interface from the proportionately huge volume of esters above it.

Since the three higher concentrations of phosphoric acid constituted a heterogeneous solution with the substrate, the equilibrium concentration of phosphoric acid in solution at any one time



should have been the same in all three cases. Hence, if the mechanism were a solution phenomenon, identical reaction curves should have been obtained for quinol and also for peroxide formation in the respective systems. This was not true when phosphoric acid was used either alone (Fig. 1, C, D) or with quinone (Fig. 3).

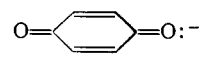
If the mechanism is not a solution phenomenon, it must be an adsorption reaction. This is confirmed by the fact that the 0.0002% phosphoric acid which appeared to be completely in solution was ineffective by itself (not shown) and had no synergic effect with quinone; the peroxide curve with quinone (Fig. 4, B), mounted as rapidly as that of a control (not shown).

In heterogeneous reactions of this type, diffusion and adsorption must precede chemical reaction. Chemical reaction is determined by the amount of reacting material adsorbed at a given moment which necessarily depends upon the free surface available.

The proposed mechanism to explain the stabilization of fat esters by phosphoric acid without and with quinone is based on adsorption and exchange reactions. In conformity with the views presently held[9] the formation of a free radical and the initiation of reaction chains may be represented by equation 1, the last term of which is the activated α-methylene group that starts the reaction chains. The same activated state of the ester molecule (the first term in 1) also facilitates the exchange reaction shown in 2. The role of phosphoric acid in such exchange reactions is well attested.[10] When the excess energy in this phosphoric acid addition compound is dissipated in the complex, this complex disassociates and regenerates phosphoric acid and an ester molecule which has now become inactivated.

This exchange reaction is limited to the surface of phosphoric acid since the acid is not appreciably soluble in the fat medium. Many activated ester molecules can become peroxide radicals in solution before they have the opportunity to be adsorbed on the acid and deactivated.

Quinone can also combine with phosphoric acid forming the phosphorylated quinone shown in equation 3. This compound probably results from the resonance of quinone to a structure similar to that shown



which can form a hydrogen bond with phosphoric acid and then rearrange to give the phosphorylated quinone. This compound could probably become doubly phosphorylated; it can be pre-

(9) E. H. Farmer, *Trans. Faraday Soc.*, **42**, 228 (1946).

(10) A. Farkas and L. Farkas, *Ind. Eng. Chem.*, **34**, 716 (1942).

pared by the reaction of phosphoric acid with quinone dissolved in an inert solvent. The resultant brown-colored substance which appears within a few hours can be freed from phosphoric acid and is insoluble in water. The conditions are such that quinol formation by reduction of quinone could not have occurred but the substance nevertheless responds to the Emmerie and Engel test, perhaps because in the alcoholic solution the free hydroxyl groups of the phosphoric acid are esterified with resulting greater resonance and increased depth of color. Reduction of ferric iron seems not to be involved. The exact reason for this behavior and the nature and properties of the complex are being studied. In the subsequent text and in the figures it is referred to as the quinol.

Because it is soluble in the fat phase, this phosphorylated quinone (the quinol) can diffuse into solution and become an exchange agent which acts in the same manner as phosphoric acid in equation 2. The reversal of this reaction gives rise to an inactivated ester molecule and the quinol. Quinone thus acts as a carrier for the exchange agent, which is thereby made soluble in the ester and can now exert an inhibiting effect throughout the medium.

The actual mechanism of the antioxygenic action of the quinol is explained by the interpretation of curve C (Fig. 3) and curve A (Fig. 4). Small amounts of peroxides appear (Fig. 4, A) before enough of the quinol has been produced to prevent their formation. The gradual increase in the quinol (Fig. 3, C) is not an artifact because the amount of peroxide formed is far too small to interfere with its determination. The rate of formation of the quinol is too slow to check the initial formation of peroxides. Once formed, the peroxides do not disappear (Fig. 4, A) although the quinol steadily increases to a constant level which depends on the extent of the adsorbing surface of the phosphoric acid and the amount of the complex formed. Peroxides neither disappeared nor increased when 0.09% phosphoric acid was present in a quinone-ester substrate containing preformed peroxides. The antioxygenic action of the quinol is therefore to prevent the formation of further peroxides rather than to react with peroxides already formed. Thus the quinol interrupts the chain mechanism through direct diversion of the original activated ester molecule.

It is obvious why phosphoric acid is an effective stabilizer in vegetable oils which contain phenolic antioxidants, why it is almost useless in animal fats which do not contain them and is also without effect in vegetable oils from which they have been removed by selective adsorption.[11]

Many heretofore anomalous reactions can be explained by this combined mechanism of adsorption, complex formation and solution. Aside from phosphoric acid, certain other acids and acidic substances have an antioxygenic action on tocopherol-containing vegetable oils whereas other acids have no such effect. Prominent among the inactive compounds are the halogen acids, nitric, boric, formic, acetic and several other organic acids as well as sodium and potassium dihydrogen phosphate and lecithin. Active acids are phosphoric, sulfuric, oxalic, malonic, citric, tartaric, malic, maleic, caffeic, pyruvic, ascorbic and hydroxyphenylaminopropionic (tyrosine) as well as calcium dihydrogen phosphate and cephalin. The effectiveness or ineffectiveness of these substances depends (1) upon their capacity to form a complex with quinone and quinol and (2) upon the relative ease with which they are themselves oxidized. Sulfuric acid has one less hydroxyl than phosphoric and should therefore not be able to interchange as freely; it is not as good a synergist as phosphoric acid. Although the halogen acids can complex with quinone the resultant product is an oxonium compound which is insoluble in fat solvents[12] and cannot act as an exchange agent.

The well-known oxidizing property of nitric acid effectively prevents it from participating in such an exchange reaction. Any complex which could be formed with the dilute acid would probably be an oxonium type similar to that of the halogen acids. Boric acid is a tribasic acid, but its hydroxyls are tied up in intermolecular hydrogen bonds,[13] and its potential ionizing properties are so slight that it cannot readily form complexes with quinone or fat molecules.

Similarly in potassium or sodium dihydrogen phosphate there are hydrogen bonds between oxygen atoms of adjacent complex anions.[13] The corresponding calcium salt is much less ionized, and mole for mole, in its un-ionized condition, it has twice the number of hydroxyl groups found in the alkali salts.

The inactivity of lecithin as a synergist is due to the internal neutralization of the phosphoric acid. In cephalin one hydroxyl is available for complex formation. Its antioxygenic action is limited by its own susceptibility to oxidation; in the presence of quinone the liberated phosphoric acid can form the lipid-soluble complex.

The various active organic acids can be considered as behaving in the same manner as the inorganic, both with and without the presence of quinone. All of these acids possess a proximal functional group such as keto, hydroxyl, halogen, amino or another carboxyl group or proximal unsaturation. Such a group facilitates hydrogen bonding by these acids; furthermore the increased repelling force due to the transmitted inductive effect of this group tends to make the oxygen donate its hydrogen to a suitable acceptor. The complex formation may also involve direct carbon to carbon bonds or carbon to phosphorus bonds. The antioxygenic action of these quinol-

(11) A. R. Moss and J. C. Drummond, *Biochem. J.*, **32**, 1953 (39).

(12) Unpublished results.

(13) L. Pauling, "The Nature of the Chemical Bond," Cornell University Press, Ithaca, N. Y., 1939.

transported acids is also dependent, in part, on their own stability toward oxidation.

Particular study has heretofore been given to ascorbic acid.[14,15] In addition to being an active organic acid, ascorbic acid is also a powerful reducing agent. It can reduce quinone to quinol and can probably also form complexes with both.

The synergic action of phosphoric acid with tocopherol[6] must likewise be reconsidered; the apparent reduction and cyclization of tocoquinone to tocopherol by traces of phosphoric acid has never been satisfactorily visualized. The nature of the analogous complex in this case is also being investigated. With an increasing understanding of these exchange reactions various chemical and biochemical conceptions may be subject to revision.

(14) Calvin Golumbic and H. A. Mattill, THIS JOURNAL, **63**, 1279 (1941).

(15) V. P. Calkins and H. A. Mattill, *ibid.*, **66**, 239 (1944).

The author is grateful to H. A. Mattill for suggesting this problem and for his help in the preparation of this paper.

Summary

The antioxygenic action of quinone with phosphoric acid in delaying fat autoxidation has been investigated. From the experimental data a scheme has been proposed, which involves both adsorption and solution reactions and which is based upon an exchange mechanism. The scheme also explains the effectiveness of various organic acids and other synergists in the stabilization of vegetable oils and fats which contain phenolic inhibitors or their oxidation products.

IOWA CITY, IOWA RECEIVED AUGUST 27, 1946

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Preparation and Properties of Serum and Plasma Proteins. VIII. The Conversion of Human Fibrinogen to Fibrin under Various Conditions[1,2]

BY JOHN D. FERRY AND PETER R. MORRISON

I.

The final step in the clotting of blood is considered to be the reaction between the proteins fibrinogen and thrombin to form a solid structure of fibrin. Many investigators have studied this process by clotting plasma, in which only about 4% of the protein is fibrinogen, and where the reaction may be complicated by the presence of other substances involved in the clotting complex (such as antithrombin), or by using fibrinogen and thrombin preparations of doubtful and unspecified purity.[3] Others have recognized the desirability of working with purified preparations of known fibrinogen content and thrombin activity, respectively.[3,4]

Various observers, even recently,[5] have questioned the enzymatic nature of thrombin. However, the fact that the amount of fibrin formed is independent of the amount of thrombin present, over a wide range,[6] and the ability of thrombin to convert over 10^5 times its own weight of fibrinogen to fibrin,[7] appear to be decisive evidence that the role of thrombin is catalytic. It has been suggested that the formation of the solid structure of fibrin does not involve any profound intramolecular changes in the fibrinogen (such as occur when globular proteins are denatured[9,10]) but merely the attachment of fibrinogen molecules together to form a network in which they retain their identity. The evidence for this view, although not yet conclusive, is, first, that the X-ray patterns of fibrin and fibrinogen are very similar,[11] and, second, that the products of partial digestion by a proteolytic enzyme of fibrin and of fibrinogen appear to be identical in solubility behavior, electrophoretic mobilities, and sedimentation constants.[12]

The conversion of fibrinogen to fibrin may thus be considered as a polymerization process, in which the "monomer" is itself a very large molecule. The polymer, fibrin, is of course usually crosslinked, since a rigid gel is formed even at

(1) This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(2) This paper is Number 47 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(3) For a critical review, see E. Chargaff, *Advances in Enzymol.*, **5**, 31 (1945); *cf.* E. Wöhlisch, *Ergeb. Physiol. expil. Pharmakol.*, **43**, 174 (1940); A. J. Quick, "The Hemorrhagic Diseases and the Physiology of Hemostasis," C. C. Thomas Co., Baltimore, Md., 1942.

(4) (a) W. H. Seegers and H. P. Smith, *J. Biol. Chem.*, **140**, 677 (1941); (b) K. Laki, *Studies Inst. Med. Chem. Univ. Szeged*, **2**, 27 (1942).

(5) W. F. H. M. Mommaerts, *J. Gen. Physiol.*, **29**, 103, 113 (1945).

(6) P. R. Morrison, in preparation.

(7) For example, in one experiment[6] 1 unit of thrombin clotted 0.18 g. of fibrinogen. Estimating the maximum weight of thrombin present from the highest prothrombin activity thus far reported,[8] 1500 units per mg., we obtain a minimum weight ratio of fibrinogen to thrombin of 2×10^5.

(8) W. H. Seegers, E. C. Loomis and J. M. Vandenbelt, *Arch. Biochem.*, **6**, 85 (1945).

(9) W. T. Astbury and R. Lomax, *J. Chem. Soc.*, 846 (1935).

(10) H. Neurath, J. P. Greenstein, F. W. Putnam and J. A. Erickson, *Chem. Rev.*, **34**, 157 (1944).

(11) K. Bailey, W. T. Astbury and K. M. Rudall, *Nature*, **151**, 716 (1943).

(12) W. H. Seegers, M. Nieft and J. M. Vandenbelt, *Arch. Biochem.*, **7**, 15 (1945); C. G. Holmberg, *Arkiv. Kemi Mineral, Geol.*, **17A**, No. 28 (1944); G. Kegeles, W. H. Seegers and J. W. Williams, unpublished experiments.