[CONTRIBUTION FROM THE NEWARK COLLEGES OF RUTGERS UNIVERSITY]

The Hydrolysis of Starch as Indicated by Congo Red[1]

BY BENJAMIN CARROLL AND JOHN W. VAN DYK

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The adsorption of congo red by amylose and amylopectin is indicated by a spectral change. At *p*H 7 the adsorption of dye is accompanied by a 21% decrease in optical density at $\lambda = 400$ mμ. A considerably larger spectral change is observed at a *p*H in the region of the *pK* of the dye. The specific adsorption depends upon the type of starch and its molecular weight. The enzymic activity of pancreatic amylase is determined by the spectral change in a starch–congo red solution. The results are compared with the usual iodine method for assaying amyloclastic activity.

During the past several years the adsorption of dyes by colloidal substrates of biochemical interest, particularly native proteins, has been investigated to reveal some of the topological features of these macromolecular substances. The chromotropic action of some proteins upon dyes has provided information regarding the number of binding sites[2] available on the colloidal particle, the free energy of binding,[3] also the rate of denaturation[4] and hydrolysis of the protein.[5]

In the present work a similar approach has been tried in the case of some starches, using chromotropic effects to differentiate and partially characterize the polysaccharides. Since the binding affinity for small molecules or ions is usually altered with changing molecular weight of the substrate, the use of suitable dyes offers the possibility of investigating the kinetics of hydrolysis of starches. This presents an additional approach to the study of the enzymic action of the α-amylases. Some results on this problem have appeared elsewhere.[6]

A preliminary study showed that the adsorption of several dyes by amylase and amylopectin was accompanied by spectral change. Some of these dyes were Benzopurpurin 4B, congo red and Alizarin Red S. In general, the adsorption was rapid and reversible. Results using congo red are reported in this work. It was found that the dye followed Beer's law reasonably well over the concentration range used and the spectral changes were sufficiently large to do quantitative work employing the usual techniques for the spectrophotometer. In addition it was observed that the dye had an inappreciable effect upon the enzymic activity of the few amylases that were tried. Thus it appeared that congo red offered the possibility of following the hydrolysis of starch continuously in the presence of dye.

Experimental

Apparatus.—All optical measurements were made in a Beckman model DU quartz spectrophotometer using cells of 1-cm. path lengths. A Beckman model G *p*H meter was used for *p*H measurements.

Materials.—A histological grade of congo red was obtained from National Aniline and used without further purification. Reagent grade chemicals were used directly for buffers. Mono- and dibasic sodium phosphate were used for the *p*H 7 buffer. Potassium acid phthalate and sodium hydroxide or hydrochloric acid were used for the buffers in the *p*H range 4.10 to 6.20.

Four samples of starch consisted of Lintner soluble potato starch, corn amylose, corn amylopectin and waxy maize.[7] The corn amylose and amylopectin were furnished originally by Dr. T. J. Schoch. The waxy maize was part of the sample used by Mindell, *et al.*,[8] and described in detail in their paper. They reported no evidence of the presence of linear components either by potentiometric titration or precipitation procedures.

The α-amylase was a crystalline preparation obtained from hog pancreatin according to the procedure of Caldwell[9] and co-workers. Only relative enzyme concentrations are given. In those cases where two or more rate curves are compared, the data were obtained within the same day using identical starch and enzyme stock solutions.

Procedures.—The binding of congo red was determined as follows: Identical quantities of buffer were added to a series of 50-ml. volumetric flasks containing a given quantity of congo red. The ionic strength of the buffer in the final solution was 0.01. A given volume of starch solution was added and the resulting solution was diluted to 50 ml. The optical density was determined shortly thereafter. Wherever deemed necessary, appropriate starch blanks were used to zero the spectrophotometer.

Two procedures were used in following the hydrolysis of starch. In procedure 1, 5-ml. aliquots of the hydrolysis mixture were removed at various time intervals and diluted in 50-ml. volumetric flasks containing 10 ml. of dye solution. The dilution of 10:1 was found sufficient to "stop" the reaction. The optical density at 400 mμ was immediately determined. In procedure 2, the dye was included in the hydrolysis mixture. In both cases the hydrolysis mixture was thermostated to $\pm 0.05°$. A water blank was used to zero the spectrophotometer. The results obtained with this method were compared in some instances with the familiar starch–iodine method. This was done by using procedure 1 and substituting I_2 reagent for dye. The final concentration of I_2 reagent was 0.0012% iodine and 0.012% KI. The optical density was measured at 620 mμ for Lintner starch and 635 mμ for amylose.[10]

TABLE I

FREE ENERGY OF BINDING OF CONGO RED AT 27°

Starch	$k_1 \times 10^{-3}$	$-\Delta F^0$, kcal.
Corn amylose	4.2	4.98
Corn amylopectin	2.0	4.53
Waxy maize	2.0	4.53
Lintner	1.3	4.28

Experimental Results and Discussion

Effect of *p*H on Solutions of Congo Red and Starch.—Figures 1a through 1f compare the ab-

(1) (a) The authors wish to thank the Research Corporation and the Rutgers Research Council for generous grants in aid of this investigation. (b) Reported in part at Meeting of American Chemical Society, Buffalo, N. Y., March 25, 1952.

(2) I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946); G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 573 (1949).

(3) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946); F. Karush and M. Sonenberg, *ibid.*, **71**, 1369 (1949).

(4) F. Haurowitz, F. DiMoia and S. Tekman, *ibid.*, **74**, 2265 (1952); also see I. M. Klotz, H. Triwush and F. M. Walker, *ibid.*, **70**, 2025 (1948).

(5) B. Carroll, *Science*, **111**, 387 (1950).

(6) B. Carroll and J. W. Van Dyk, *ibid.*, **116**, 168 (1952).

(7) The authors are indebted to Prof. Mary L. Caldwell for her generous gift of crystalline pancreatic amylase and the starch samples used in this work.

(8) F. M. Mindell, A. L. Agnew and M. L. Caldwell, THIS JOURNAL, **71**, 1779 (1949).

(9) M. L. Caldwell, M. Adams, M. Kung and G. C. Toralballa, *ibid.*, **74**, 4033 (1952).

(10) See W. W. Smith and J. H. Roe, *J. Biol. Chem.*, **179**, 53 (1949).

sorption spectrum of 1.6×10^{-5} M congo red in the presence and absence of 0.1% Lintner starch in the pH range of 4.1 to 7.0. It is evident from the curves that the apparent pK of congo red is decreased by the presence of starch. Measurements made at pH 2 showed no appreciable difference in the spectrum of congo red in the presence of Lintner starch. Since solutions of congo red are unstable at this pH, the accuracy was very poor. At pH 9 and 11, the spectra were very similar to those obtained at pH 7.

The graphs indicate that congo red complexes with starch and that this action is accompanied by spectral shifts that decrease in magnitude as the pH of the system is increased from 4.1 to 7.0. At pH 7.0 the absorption minimum remains unaltered at 400 mμ but is decreased 21%.

It is interesting to note the pH dependence is similar to that found for congo red when it is adsorbed by ovalbumin or serum albumin.[4] The major difference between the two systems is that the decrease in apparent pK of the dye is larger in the protein system. This suggests that the mechanism of binding may be similar in both cases.

Since it was desired to seek a method where the enzymatic activity of pancreatic amylase could be assayed in the presence of the dye, all subsequent work was done at pH 7, this being in the region of the pH optimum of the amylase. It is obvious from the results in Fig. 1 that the precision of the dye assay method can be improved considerably if aliquots of the hydrolyzing starch mixture are added to the dye buffered in the neighborhood of the pK value of congo red which is about 4.1.

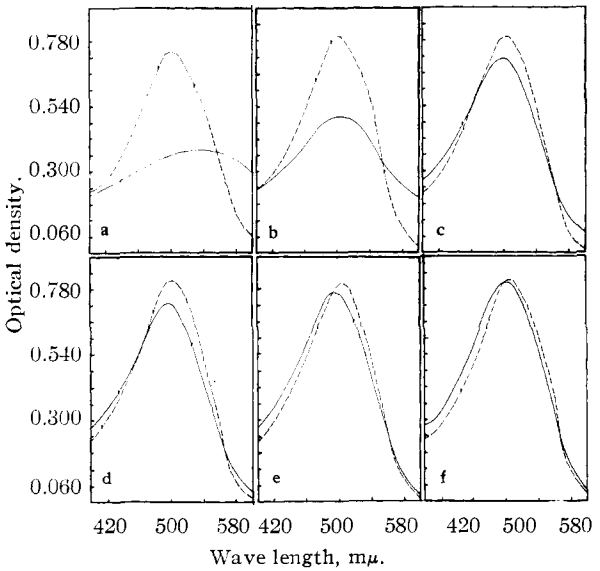


Fig. 1.—Optical density of 1.6×10^{-5} M congo red in the presence and absence of 0.1% Lintner starch. Solid line represents dye alone. Broken line represents system of dye plus starch at pH: (a), 4.10; (b), 4.45; (c), 4.85; (d), 5.35; (e), 6.20; (f), 7.00.

Adsorption of Congo Red at pH 7.0.—It was found that congo red followed Beer's law within ±3% over the concentration range used here provided that salt concentration of the dye solution was kept low and constant. Upon the addition of excess starch the extinction coefficient of congo red attained a constant value regardless of the type of starch used. It was assumed that the constant extinction coefficient indicated that all the dye was bound. At 400 mμ the ratio of the extinction coefficient of bound to unbound dye, $E_{ads.}/E_{free}$, was 0.79.

It was thus possible to summarize the effect of starch upon congo red in terms of "r," the number of dye molecules bound per glucose unit, as a function of (A), the unbound dye concentration. The results are given in Fig. 2 for corn amylose, corn amylopectin, waxy maize and Lintner starch. It should be pointed out that these results were obtained mainly by keeping the total concentration of dye constant and varying the concentration of the starch in the system. This procedure was employed because optical densities thus obtained were used directly to determine the optimum concentration of starch for hydrolysis experiments. Probably better precision for adsorption data would have resulted if the starch concentration had been kept constant and the total dye concentration varied.

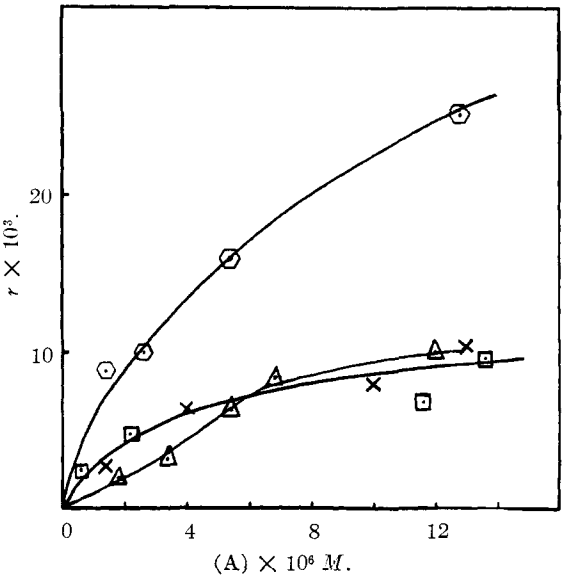


Fig. 2.—The adsorption of congo red at 27°; pH 7.0; moles of dye bound per glucose unit, r *vs.* free dye concentration: ⬡, corn amylose; □, corn amylopectin; ×, waxy maize; △, Lintner starch.

Figure 2 shows that the specific absorption of Lintner, corn amylopectin and waxy maize are about the same and are significantly smaller than the specific adsorption for corn amylose. For low concentrations of free dye, the binding affinity for congo red may be given the series: corn amylose > corn amylopectin = waxy maize > Lintner.

A thermodynamic treatment of the binding data is justifiable since it was found that the binding process was a reversible one. If it is assumed that there are n binding sites available per glucose unit with an intrinsic association constant k and if it is further assumed that there is no interaction among

the bound ions, an equation[2]

$$[r/(A)] = kn - kr \quad (1)$$

may be considered.

The application of this formula implies that congo red exists in monomeric form. Since this is probably the case for congo red at infinite dilution, an extrapolation of $[r/(A)]$ *vs.* r or (A) results in a relationship applicable to this system. According to equation 1

$$(A) \xrightarrow{\lim} 0 \; [r/(A)] = nk = k_1 \quad (2)$$

In Table I, the free energy of binding corresponding to the extrapolated values have been calculated[11] using the equation

$$\Delta F^0_1 = -RT \ln k_1 \quad (3)$$

It should be noted that the binding data are not any more reliable than about ±10% because they were obtained as a difference between two relatively large numbers. Nevertheless, it can be seen that the linear component of corn starch amylose has the highest binding affinity for congo red. This was not entirely unexpected since the branching occurring in amylopectin would tend to prevent close adlineation between congo red and starch. The identical binding values obtained for corn amylopectin and waxy maize indicate structural similarity as has been observed by others.[12] Lintner starch which is a partially degraded mixture of amylopectin and amylose has the least binding affinity, undoubtedly due to its low molecular weight. This will be evident from the following hydrolysis studies.

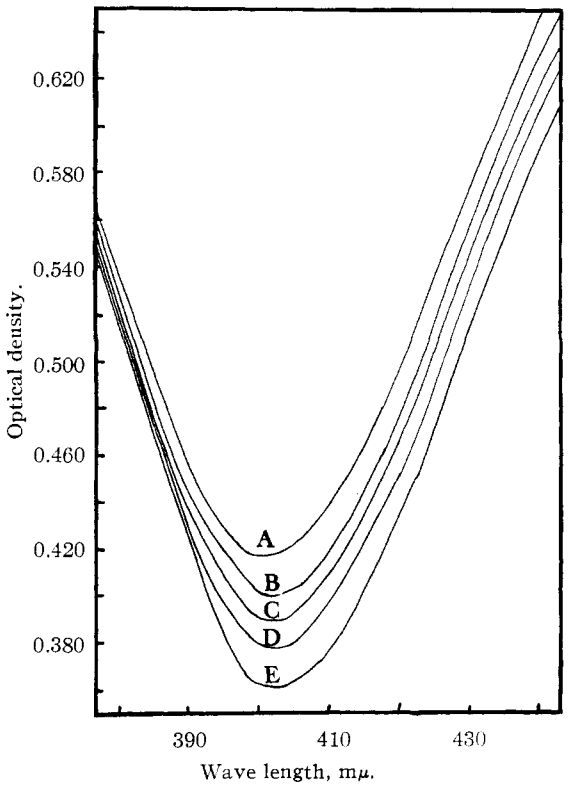


Fig. 3.—Optical density at 400 mμ of congo red when added to aliquots of a starch–pancreatic amylase solution at various time intervals. Final dye–hydrolysate mixture contained 2.4 × 10^{-5} *M* congo red, 0.03% Lintner starch, 0.008 *M* NaCl and phosphate buffer, (*p*H 7, μ 0.02); temperature 29.5°: A, dye alone ($t = \infty$); B, t = 40 minutes; C, t = 27 minutes; D, t = 17 minutes; E, t = 0 minutes.

(11) If the dye remains aggregated at infinite dilution the calculated free energies will not be significant although they may indicate the relative binding capacities. It has been shown by Robinson and coworkers (*Trans. Faraday Soc.*, **35**, 771, 780 (1939)) using conductivity and osmotic pressure measurements that congo red has a maximum aggregation number of 4.0 at 4 × 10^{-4} *M*. This number decreases to 2.1 at 2.5 × 10^{-5} *M*. No measurements have been reported for lower concentrations of dye. The extrapolations for obtaining k_1 in equation 2 have been based on binding data where (A) is in the neighborhood of 10^{-4} *M*. Our spectral studies at this concentration appear to indicate that most if not all of the dye is in monomeric form.

Hydrolysis Studies

Assay of Amylase Activity with Congo Red.—Preliminary investigation showed that the hydrolysis of the four starches discussed in the previous section diminished their ability to bind congo red. It was found that the adsorption as well as the desorption process was rapid. For example, it was possible to inoculate a dye–starch mixture with a sufficiently high concentration of pancreatic amylase so that the starch almost completely lost its affinity for dye in less than one minute. Thus the breakdown of starch into dextrins and sugars could be followed in two ways as described in the Experimental section. The concentration of starch and dye in a reaction mixture was limited to that range where an over-all precision of about ±5% was obtained. Use of the spectrophotometer as a differential instrument should improve the precision considerably in following the course of hydrolysis.

Figure 3 shows the typical spectral changes which occur in a congo red–hydrolysate solution during the course of a reaction. The wave length of the absorption minimum does not depend upon the type of starch or molecular weight of the starch. The optical density can be related to the extent of the hydrolysis by keeping the spectrophotometer set at λ 400 mμ.

Under similar conditions of temperature and substrate concentration the time required to reach a given point in the hydrolysis as indicated by the optical density should be inversely proportional to the enzyme concentration. To test this assumption, the following experiment was performed: Four hydrolysis mixtures containing enzyme concentrations in the ratio 1:2:4:10 were thermostated at 29.5°. Each mixture contained, in addition to the enzyme, 0.3% Lintner starch, 0.008 *M* NaCl, and phosphate buffer (*p*H 7, μ 0.02). At various time intervals, 5-ml. aliquots of the hydrolysate were diluted 1 to 10 in a flask containing sufficient congo red to make the final dye concentration equal to 2.4 × 10^{-5} *M*. The optical density at 400 mμ was then measured and plotted as a function of time in Fig. 4. The time at which the given points were reached were interpolated from Fig. 4 and plotted as a function of the reciprocal of the enzyme concentration in Fig. 5. The linearity of the plot confirms the assumption and makes it possible to use the dye method to assay the enzyme. The

(12) H. H. Schopmeyer, G. E. Felton and C. L. Ford, *Ind. Eng. Chem.*, **35**, 1168 (1943).

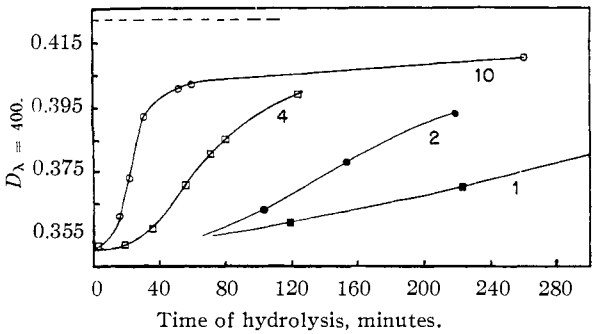


Fig. 4.—Hydrolysis of 0.3% Lintner starch with pancreatic amylase at 29.5° as followed by a change in optical density of a solution of 2.4×10^{-5} *M* congo red containing aliquots of the hydrolysate (0.008 *M* NaCl and phosphate buffer *p*H 7; μ 0.02 was present in all hydrolysis mixtures); relative amylase concentration as indicated. Broken line indicates optical density of congo red in absence of starch.

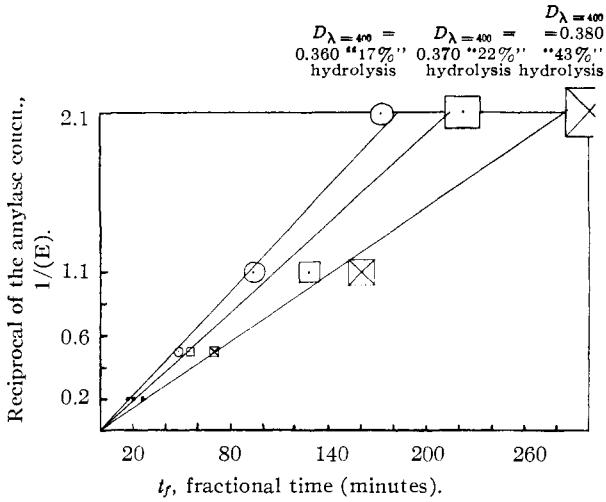


Fig. 5.—Reciprocal of the amylase concentration 1/(E) *vs.* the time t_f required for hydrolysate–dye solution to attain a given optical density at 400 mμ. Plot based on results in Fig. 4.

results obtained when congo red was included in a hydrolysate of Lintner starch and pancreatic amylase were similar to those previously obtained[6] and are summarized in Fig. 6. Inclusion of the dye in the reaction mixture made for somewhat better precision because of greater number of spectrophotometric observations that could be taken; further there was no error introduced in stopping the reaction.

Inhibiting Effect of Dye.—Several runs were made with 0.1% solutions of amylose and waxy maize to determine the effect of the presence of 2×10^{-5} *M* congo red in a hydrolyzing mixture. Within the experimental error of about ±5%, no inhibition of the enzymatic hydrolysis was observed. To test the inhibiting effect of the dye further, two identical reaction mixtures containing pancreatic amylase, 0.1% corn amylose, 0.025 *M* KCl and phosphate buffer (μ 0.02, *p*H 7.0), were prepared. Sufficient congo red was placed in one reaction mixture to make the final concentration 3

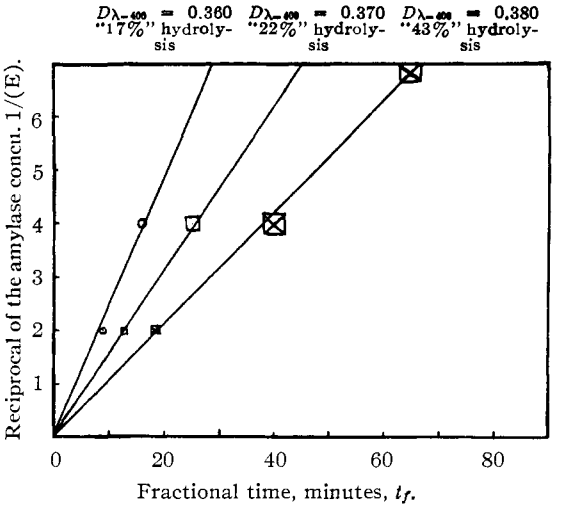
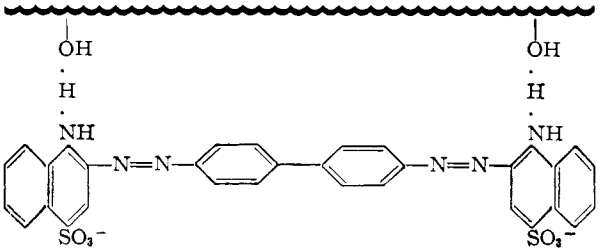


Fig. 6.—Relation of reciprocal of the enzyme concentration 1/(E) to t_f for the hydrolysis of 0.03% Lintner starch by pancreatic amylase in the presence of 2.4×10^{-5} *M* congo red.

$\times 10^{-4}$ *M*. This was about 10 times the final concentration of dye normally used in the dye assay method. At various time intervals, aliquots of both hydrolysate mixtures were diluted 1 to 10. Sufficient congo red was added to the dye free hydrolysate to make the final concentration of congo red equal to 3×10^{-5} *M*. When the times required to reach the same optical densities were compared, it was found that the system containing the dye initially was inhibited about 30%.

Because it was not possible to determine the number of dye molecules adsorbed at 3.0×10^{-4} *M* congo red in a 0.1% amylose solution it is difficult to correlate quantitatively the inhibiting effect with increased concentration of dye. However, some qualitative estimates may be made: At a total concentration 3×10^{-5} *M* congo red in 0.1% amylose "*r*" had a value of 5×10^{-3}, *i.e.*, on the average every two hundred glucose units contained one dye molecule. Each dye molecule may render several glucose units inaccessible to the enzyme. It appears that the two amino groups in congo red form the foci of attachment to the starch *via* hydrogen bonding[13] as



Because of the mutual electrostatic repulsion of its two sulfonic acid groups, the congo red anion is probably in a flat extended state.[4] If it is assumed that a single adsorbed dye molecule renders some six glucose units inaccessible it is not difficult to see why a value of 30% inhibition was obtained

(13) T. Vickerstaff, "The Physical Chemistry of Dyeing," Interscience Publishers, Inc., New York, N. Y., 1950.

for a total dye concentration of 3×10^{-4} M during the hydrolysis of 0.1% amylose. It should be mentioned that spectrophotometric observation of congo red in the presence of relatively high concentrations of crystalline pancreatic amylase ($\sim 0.01\%$) at pH 7 indicated an absence of complex formation. This is in keeping with the general observation that with the exception of serum albumin most native proteins bind anionic dyes poorly at this pH.[14] It appears that the study of the effect of enzymes on partially "coated" substrates where the coating consists of adsorbed dyes may prove a useful approach to the study of the mechanism of enzymic action. A similar approach was used in the pepsin–serum albumin reaction[15] in an attempt to ascertain the existence of the type of long range forces postulated by Rothen.[16]

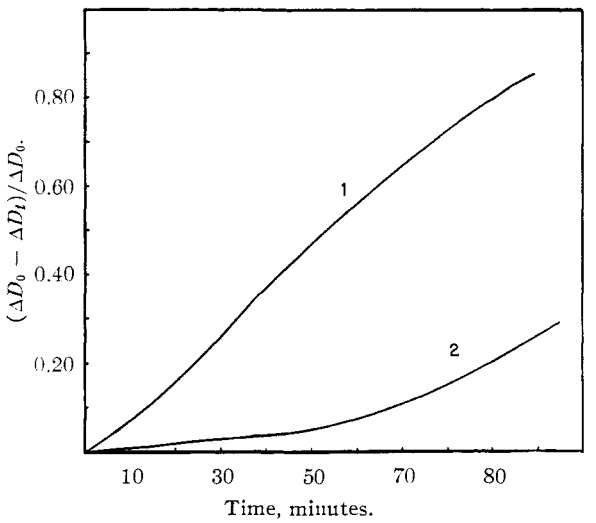


Fig. 7.—Hydrolysis of corn amylose by pancreatic amylase: "Fraction of reaction completed" $(\Delta D_0 - \Delta D_t)/\Delta D_0$ measured by the iodine and congo red spectral shifts as a function of the time. Same hydrolysate was used for both curves: 1, iodine method, λ 635 mμ; 2, dye method, λ 400 mμ; temperature 29.5°.

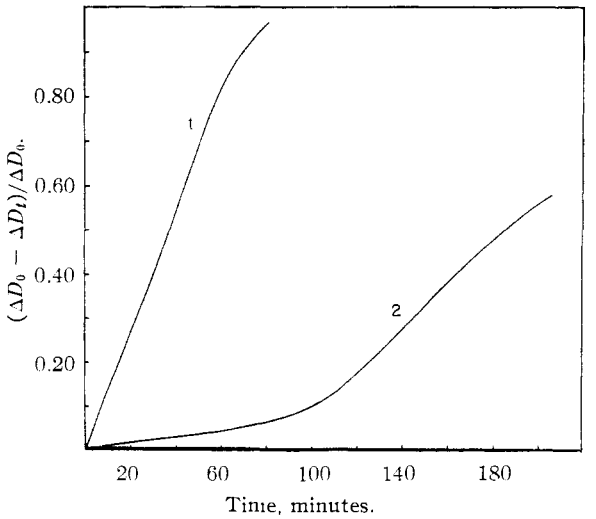


Fig. 8.—Hydrolysis of Lintner starch by pancreatic amylase: "Fraction of reaction completed," $(\Delta D_0 - \Delta D_t)/\Delta D_0$ as measured by the iodine and dye spectral shifts as a function of the time. Same hydrolysate was used for both curves: 1, iodine method, λ 620 mμ; 2, dye method, λ 400 mμ; temperature 29.5°.

(14) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).

Comparison of Dye and Iodine Methods for Amyloclastic Activity.—It is possible to compare the two methods under identical conditions by adding portions of a hydrolyzing mixture to an iodine solution and to a congo red solution as described in the Experimental section. The fraction of the reaction completed as measured by the two methods may be plotted as a function of the time. The fraction of the reaction completed may be defined as

$$\tau = \frac{\Delta D_0 - \Delta D_t}{\Delta D_0}$$

where $\Delta D_0 = D_0 - D_r$ and $\Delta D_t = D_t - D_r$. Here D_0 is the optical density, at the appropriate wave length, of hydrolysate plus iodine or dye at zero time, and D_t is the optical density at time t. The optical density at the appropriate wave length of iodine or dye alone is D_r. The results comparing the iodine and congo red methods for amylose and Lintner starch are indicated in Figs. 7 and 8, respectively.

It is obvious from these figures that the starch–iodine color changes much more rapidly than the starch–congo red, the difference being most marked in the early stages of the hydrolysis. This indicates the dye is bound by dextrins which are smaller than those necessary for the formation of a starch–iodine complex. It will be seen from Figs. 7 and 8 that at $\tau = 0.3$ the ratio of the time for iodine to congo red is 1:3. In the case of Lintner starch the ratio is 1:6. An approximation may be made of the relative length of substrate required for producing a spectral effect upon congo red from the fact that it took about 5 times as long at a given concentration of α-amylase for the Lintner starch reaction to go to completion using congo red in place of iodine. If chains of 30 glucose units or longer are required for a blue color with iodine,[16] roughly a fifth this length may be taken as the chain length requirement for a polysaccharide to affect the color of congo red under concentration conditions as prevailed in the experiment shown in Fig. 8. A somewhat longer chain is required if the results for amylose in Fig. 7 are considered. It is of interest to note that the point at which a hydrolyzed starch just fails to yield a red color with iodine still produced a marked spectral change with congo red. This would make the minimum chain length requirement for adsorption of congo red less than ten glucose units.[17] However, it should be stated that concentration of substrate and dye are important variables in a chromotropic effect.

The data in Figs. 7 and 8 show that congo red may be of particular value as an indicator for following the hydrolysis (or synthesis) of dextrins which are practically achroic with iodine. Also the fact that the amyloclastic rates are reduced several fold as measured with congo red may be significant in increasing

(15) B. Carroll, *Ann. N. Y. Acad. Sci.*, **12**, 152 (1950).
(16) A. Rothen, THIS JOURNAL, **70**, 2732 (1948).
(17) M. A. Swanson, *J. Biol. Chem.*, **172**, 825 (1948).

the sensitivity of the method frequently used to characterize an amylase, namely, the determination of the amyloclastic to saccharenogenic ratio.[18]

(18) W. J. Whelan and H. Nasr, *Biochem. J.*, **48**, 415 (1951).

Acknowledgment.—The authors wish to thank Mrs. Van Dyk for her assistance in the laboratory and for her aid in the preparation of this manuscript.

[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

Measurements of the Density, Composition and Related Unit Cell Dimensions of Some Protein Crystals[1a]

BY BARBARA W. LOW AND F. M. RICHARDS[1b]

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Some measurements of composition, density and unit cell size are reported for crystals of β-lactoglobulin, dimer albumin and insulin. These measurements have been made for the "wet," air-dried and vacuum dried states and at other points over a range of relative humidity. The reliability and correlation of the data is discussed. Since the measurements of the composition, density and unit cell volume are all independent, the errors involved in each are unrelated. The partial specific volumes of water and protein in the dimer albumin crystals over the range 13 to 55% water have been derived. These values are not equivalent to comparable data on β-lactoglobulin reported by McMeekin. The significance of this discrepancy is discussed, it has not yet been elucidated.

Introduction

Detailed studies of the properties of protein crystals usually require preliminary measurements of their composition, density and unit cell size. From these data values for the protein molecular weight may be obtained, and the effects of immersion in a variety of media may be calculated. Measurements of protein crystal density and composition present special problems arising from the rapid loss of solvent of crystallization which occurs when the crystals are removed from their mother liquor, and the well known swelling, shrinking and permeability properties in media of different composition.

It is the purpose of this paper to report some studies on single protein crystals in which the only significant components were protein and water. The data are principally for bovine β-lactoglobulin and human serum mercaptalbumin mercury dimer (dimer albumin) crystals. Some incomplete results on acid insulin sulfate crystals also are included. The problems involved in the proper correlations of these measurements and the related concept of "water of hydration" are discussed.

Experimental

Preparation of Crystals.—In this study, large well developed, single crystals of purified protein preparations were required, especially for the composition and density measurements. Techniques were employed and precautions taken to ensure slow crystal growth, and, as far as possible, equant development. The methods used are described below.

Dimer Albumin.—The protein used was a 5 times recrystallized sample of the dimer prepared from a large lot of Squibb 352-RR Fraction V which had been rejected for clinical use because of pyrogen content. The Fraction V had originally been prepared by Method 6 as developed in this Laboratory.[2] The fraction was crystallized as the mercury dimer by the method of Hughes.[3] A sample of the final preparation, dialyzed against cysteine to remove the mercury, indicated an SH content of 0.98 mole of SH/mole of albumin. The dimer appears to be quite stable in a 10% salt-free solution. Thus since the dimer crystals are much less soluble under these conditions than those of the monomer and as the two crystalline forms are quite different, the crystals obtained may be assumed to consist wholly of the dimer form.

Preparative Procedures. (a) **Method of Lewin.**[4]—The technique described by Lewin was employed essentially unchanged. Large crystals were sometimes, but not always, obtained. The results are not predictable. (b) **Vapor Phase Addition of Precipitant.**—A 10% solution of dimer albumin in water was centrifuged to remove any particulate matter. A test-tube containing this clarified solution was placed in a larger tube holding an equivalent volume of aqueous methanol at a concentration somewhat higher than that required to cause crystallization in the protein mixture. The larger tube was tightly stoppered and the whole unit kept at 0° for several weeks. Diffusion of methanol from the outer liquid into the protein solution occurred through the vapor phase, and a gradually changing gradient of precipitant concentration was thus set up. Although small crystals frequently grew at the surface of the protein solution, large single crystals were often obtained in the body of the tube. This method appeared to give more consistent results than method (a). (c) **Use of Gelatin Gels.**—In an attempt to prepare crystalline derivatives of dimer albumin using barium hydroxide, Lewin[4] obtained rigid gels in which particularly well formed single crystals[5] grew, isolated from each other throughout the medium.[6] Comparable conditions were therefore simulated for the pure protein. A solution containing 10% dimer albumin, 1% gelatin and 5% methanol was allowed to stand at 0°. Within 12 hours the mixture was sufficiently rigid to resist pouring. After two weeks, large single crystals appeared throughout the gel. Although showing a marked tendency to twin on the (001) face, the crystals were optically and morphologically identical with those obtained by other methods. The complete absence of gelatin from these crystals has not been demonstrated. They have not, therefore, been used for quantitative measurements in this investigation.

β-Lactoglobulin.—Armour crystalline β-lactoglobulin was used without further purification. This protein has been adequately characterized in the literature and no attempt

(1) (a) This work has been supported by the Eugene Higgins Trust, by grants from the Rockefeller Foundation, the National Institutes of Health, by contributions from industry, and by funds of Harvard University. (b) This work was carried out while one of us (F.M.R.) was an Atomic Energy Commission Predoctoral Research Fellow in the Biological Sciences, and is reported in detail: F. M. Richards, Ph.D. Thesis, Harvard University, 1952.

(2) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(3) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).

(4) J. Lewin, *ibid.*, **73**, 3906 (1951).

(5) B. W. Low, *ibid.*, **74**, 4830 (1952).

(6) The observation recalls the use of gels (slow diffusion) in the preparation of large single crystals of sparingly soluble inorganic compounds; *cf.* Chamot and Mason, "Handbook of Chemical Microscopy," Vol. I, 2nd Edition, John Wiley and Sons, Inc., New York, N. Y., 1947, p. 342.