Reports

Protein Spherulites

We have recently observed that the crystal habits of the enzyme carboxypeptidase (*1*) differ according to the protein concentration of the mother liquor. At low protein concentrations a branching, sheaflike crystal morphology is observed, a typical example of spherulitic crystal growth. At high protein concentration, the classical, polyhedral crystals appear. The dependence of crystal form on protein concentration adds another parameter for study of the crystallization of this and possibly of other proteins. The existence of spherulitic behavior, which has been well studied in a number of macromolecular model systems, may have new implications for the interpretation of the biophysical organization of crystalline proteins.

Carboxypeptidase is a zinc metalloenzyme (*2*) of molecular weight 34,300 (*3*), containing one atom of zinc per molecule of protein; even though it is firmly incorporated into the molecule it can be removed and restored with concomitant loss and restoration of function, but apparently without physical change in the molecule (*4*). The primary structure consists of a single polypeptide chain of 310 amino acid residues (*5*), hydrogen bonded to form the helical secondary structure, according to the presently accepted views of protein structure (*6*). The density of 1.33 and the globular shape, denoting close packing, may result from folding of the molecule (*6*). The protein has an isoelectric point at *p*H 6.0 and a net negative charge in the region of *p*H 8.0, where the crystallizations described here were carried out (*3*). Although it is soluble to the extent of 20 to 30 mg/ml in 1 *M* NaCl, it is highly insoluble in distilled water. It is this insoluble character of the protein which permits its crystallization at the low protein concentrations and which promotes spherulitic crystal growth.

Carboxypeptidase was prepared from beef-pancreas acetone powders (*7*) by the method proposed by Allan, Keller, and Neurath (*8*). The recrystallized protein was homogeneous, as was demonstrated by ultracentrifugation and electrophoresis. For crystallization, the protein was dissolved in 1*M* NaCl and dialyzed in Visking-Nojax dialysis bags (*9*) in a rotary dialyzer at 4°C against successively decreasing salt concentrations, buffered at *p*H 8.0 with 0.02*M* sodium veronal. The speed of agitation and the rate of decrease in salt concentration were identical in all experiments.

The various crystal habits of carboxypeptidase observed in this study are shown in Fig. 1, arranged in descending order of concentration of protein (in milligrams per milliliter) of the solution employed for crystallization. Figure 1*A* shows a large, polyhedral crystal, typical of those obtained at 30 mg/ml. At concentrations of 10 mg/ml, the classical, polyhedral crystals, shown in Fig. 1*B*, are seen. These crystals consistently appear at salt concentrations of from 0.3 to 0.5*M*. At protein concentrations of 5 mg/ml, a mixture of spherulitic sheaf crystals and of the classical polyhedral morphology appears (Fig. 1*C*). At a concentration of 2 mg/ml, spherulitic crystals are obtained exclusively (Fig 1*D*). Characteristically, these crystals exhibit a typical birefringent pattern, as shown in Fig. 1*E*, under crossed Nicol prisms. By means of a gypsum plate, the larger index of refraction was found to be oriented along the radius of the spherulite, a "positive" orientation as defined by convention (*10*). In contrast to the polyhedral form, these crystals appeared consistently at salt concentrations between 0.05 and 0.1*M*. The spherulitic and polyhedral crystals, when redissolved, can be interconverted by either diluting or concentrating the protein solution to the appropriate protein concentration.

Figure 2 demonstrates the morphological details of the spherulitic crystal growth shown in Fig. 1, *D* and *E*. Figure 2 demonstrates the aggregation of the fibrous subcomponents (Fig. 2*A*) into the incompletely assembled spherulite (Fig. 2*B*), and into a spherically symmetrical complete spherulite (Fig. 2*C*.). Figure 2*D* represents branching overgrowth of a spherulitic aggregate, and Fig. 2*E*, the morphological detail of this preparation, obtained from a solution of 1 mg of protein per milliliter. The complete spherulite in Fig. 2*C* is shown again in Fig. 2*F* as seen under crossed Nicol prisms and exhibiting the characteristic birefringent pattern.

In neither mode of crystallization did the presence or absence of zinc in carboxypeptidase affect the gross crystal morphology. With the native and the metal-free derivative of the enzyme, or with the enzymes reconstituted with Fe^{++}, Mn^{++}, Co^{++}, and Ni^{++}, virtually identical types of crystal habits are obtained. These metal ions have been shown to substitute for zinc in binding to the apocarboxypeptidase with concomitant restoration of function (*11*).

The phenomenon of spherulitic crystal growth has received increasing attention ever since the discovery that this is the normal mode of crystallization in a number of synthetic and natural polymers. Spherulites were first observed by Bunn and Alcock in synthetic polyethylene in 1945 (*12*) but are now known to occur in a variety of substances, including gutta-percha, natural rubber, sphingosine (*13*), polyethylene, polymethylene, polyamides, and polyurethane (*14*). Spherulites of carbonates, sulfur, and resorcinol have also been seen, though this is not a common mode of crystallization in such simple substances (*14*).

The gross morphology and fine structure of these crystals have been discussed extensively (*13–15*). The typical birefringent pattern, a bright sphere with a dark Maltese cross whose arms lie in the direction of the axes of polarizer and analyzer, is easily recognized in polymers crystallized from the melt. Extensive studies with light (*16*) and electron microscopy (*14*) have shown the underlying structure to be wheatlike sheaves of crystallites which approach a spherically symmetrical aggregate.

Thus, the spherulitic crystal form is the normal mode of crystallization in

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Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to Contributors" [*Science* **125**, 16 (1957)].

these polymer systems. X-ray, electron diffraction, and birefringent patterns of polymers indicate that the molecules are helically wound, with the long axis of the helix along the radius of the fibrous crystallite (*14*). The spherulites of polyamides are consistently positive, and a tightly wound helix has been proposed as the model for the molecular orientation (*17*). The helix extends along the fiber axis in such a way that the long axis of the molecules is tangent to the fiber axis. Hydrogen bonding is thought to take place in planes parallel to the fiber radius.

In carboxypeptidase, the spherulitic form, analogous to that of polymers, has been observed only when the enzyme is crystallized from dilute solutions of protein. Electrostatic, covalent, and hydrogen bonds as well as weak van der Waal's forces are, of course, all known to contribute crucially to the configuration of polymers and proteins (*6*). It is not understood whether any one of these forces governs and dominates spherulitic fine structure or whether this is due to a critical balance between them.

It is well known that the environmental conditions (solvents, impurities, ionic strength, *p*H, and so on) under which crystallization takes place, alter the crystal habits of given enzymes—among other proteins—significantly (*18*). In addition, enzymes exhibiting specificity toward the same substrate but obtained from different biological species also vary markedly in gross crystal morphology (*18*). The concentration of protein in the solution in which crystallization occurs should be considered in assigning significance to observed differences in crystal forms. The significant effect of protein concentration on the crystal form of carboxypeptidase suggests that this parameter may also be responsible, in some instances, for variations in morphology currently attributed to other factors (*18*). The data reported here demonstrate conclusively that crystal morphology in this system can be reversibly altered, at will, from a spherulitic to a polyhedral form without detectable changes in enzymatic function.

It is characteristic that polymers, in crystallizing as spherulites, extend out along the long axis of the fibrous units for some distance. The appearance under crossed Nicol prisms is explained on the basis of spherically symmetrical aggregates of index ellipsoids, with the long axis either parallel or tangential to the spherulite radius (*10*). The packing and folding of the polypeptide chains of the individual protein molecules—greater than that of synthetic polymers—suggests that the protein molecules span a much shorter distance along the axis of the fibrous unit. It may be postulated that the respective axes of the protein molecules, corresponding to the larger index of refraction, line up parallel to the radius of the fiber and in colinear relationship to each other. In this manner the protein would achieve a spherulitic morphological pattern and consequent birefringent properties equivalent to those of the polymer crystal.

The implications of spherulitic morphology to fine structure of synthetic polymers may thus be extended to proteins. Therefore, inferences may be drawn concerning the orientation of the protein molecule in the crystal lattice. Carboxypeptidase may serve as a

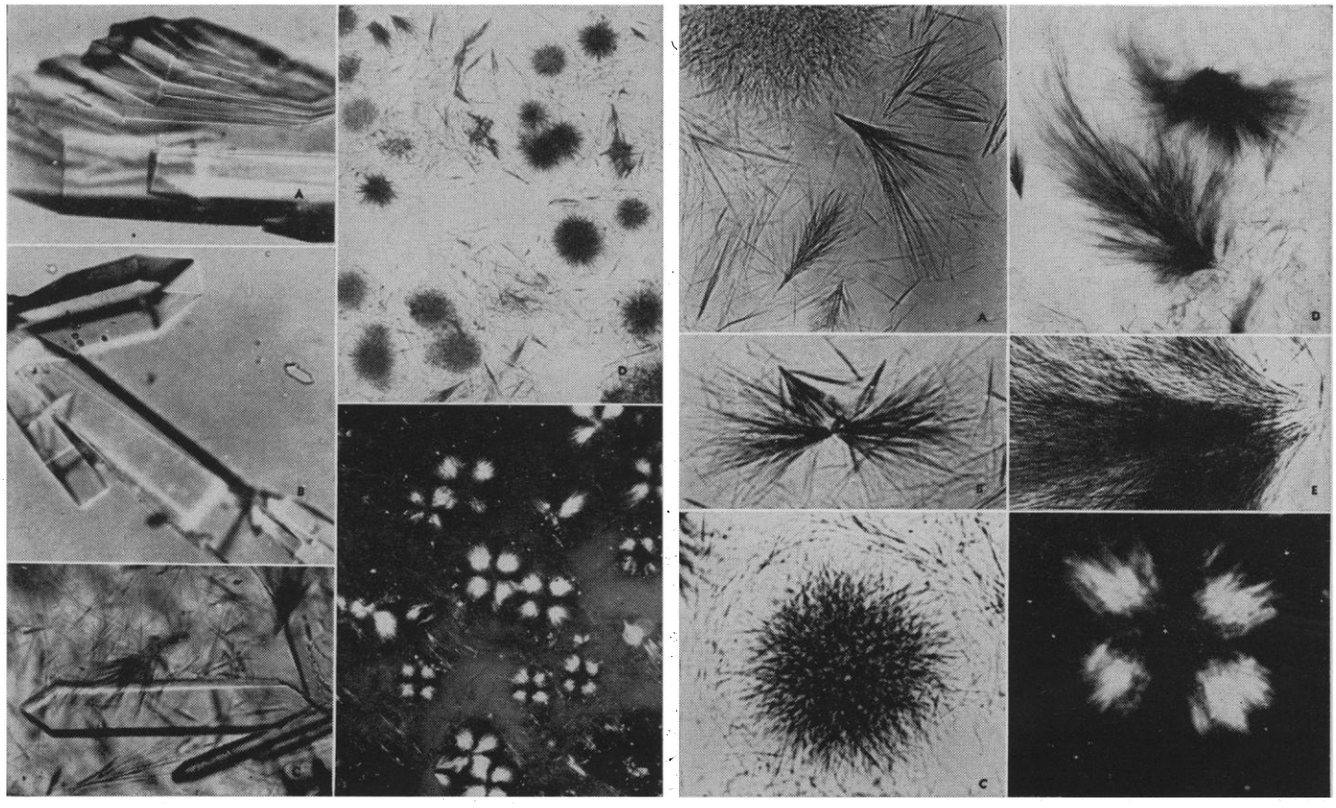

Fig. 1 (left). Crystal forms of carboxypeptidase. All concentrations given are in milligrams of protein per milliliter of mother liquor. (*A*) Large polyhedral crystal, 30 mg/ml (about × 25). (*B*) Classical, polyhedral crystals, 10 mg/ml (about × 50). (*C*) Combination of spherulites and polyhedral crystals, 5 mg/ml (about × 75). (*D*) Spherulites, 2 mg/ml (about × 50). (*E*) Appearance of preparation in *D* under crossed Nicol prisms (about × 50). Fig. 2 (right). Details of spherulitic crystal growth. (*A*) Small branched crystallites. (*B*) Partially assembled spherulite. (*C*) Spherically symmetrical, complete spherulite. (*D*) Hairy overgrowth occurring at a concentration of 1 mg of protein per milliliter. (*E*) Detail of *D*. (*F*) Appearance of complete spherulite in *C* under crossed Nicol prisms (about × 98).

prototype of a more general phenomenon in the mode of crystallization of proteins. This conjecture can be readily verified (*19*).

J. E. COLEMAN
B. J. ALLAN
B. L. VALLEE
Department of Medicine, Harvard Medical School, and *Peter Bent Brigham Hospital, Boston, Massachusetts*

References and Notes

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19. This work was supported by grants from the Nutrition Foundation, the National Institutes of Health of the Department of Health, Education, and Welfare [No. 3117(c)] and the Howard Hughes Medical Institute. We are indebted to R. V. Coleman of the department of physics, University of Illinois, Urbana, for helpful discussions and suggestions. The senior author is a fellow in medical science of the National Academy of Sciences–National Research Council.

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Virulence Transformation of a Trichomonad Protozoan

Abstract. Treatment of an avirulent strain of *Trichomonas gallinae* with a cell-free homogenate of a virulent strain resulted in enhanced virulence as evidenced by the size of lesions produced in mice. Addition of deoxyribonuclease to the homogenate cell mixture blocked the transformation.

Marked differences in virulence in strains of *Trichomonas gallinae*, a flagellate parasite of columbiform and galliform birds, can be demonstrated readily in natural hosts (*1*) and in laboratory mice (*2*). It seemed feasible to determine whether transformation of the virulence of a relatively nonpathogenic strain to enhanced virulence might be accomplished by treating the cells with cell-free material from a highly pathogenic strain, although, as far as can be ascertained, such transformation of protozoans has not been reported previously.

Differences in pathogenicity among strains of *T. gallinae* from axenic cultures can be evaluated quantitatively by comparison of the mean volumes of 5-to-6 and 12-to-14 day lesions produced in mice by subcutaneous flank inoculation of 7.5 to 9.0 $\times$ 10^5 parasites in 0.5 ml of medium (*3*). This assay for virulence was employed in the present investigation.

Two strains of *T. gallinae* were used: the very pathogenic Jones' Barn strain (JB), isolated in axenic culture from a liver abscess of a pigeon in the summer of 1958, and the relatively nonpathogenic Lahore strain (YG), isolated in a similar culture from the mouth of an apparently healthy pigeon in the early fall of 1956. Both experimentally infected birds came from Robert Stabler. It must be noted that in the long series of transfers on fluid thioglycollate (FT) with 1 or 5 percent normal horse serum (FTS), YG strain has become further attenuated in pathogenicity and will be referred to henceforth as the YGA strain.

Strain JB flagellates were transferred from FTS to CPLM medium (cysteine, peptone, liver influsion, maltose) with 5 percent serum but without agar (CPLMNA), on which they were maintained for three transfers. Twenty-four-hour cultures (total of 6 liters) from the fourth transfer were centrifuged at 2000 rev/min for 10 minutes at 5°C, and the centrifugate was washed twice with Earle's balanced salt solution containing penicillin and dihydrostreptomycin. The washed centrifugate was suspended in 5 ml of sterile 0.154*M* sodium chloride–0.01*M* sodium citrate and frozen immediately. (Standard sterility tests for the presence of microorganisms other than the trichomonads gave uniformly negative results.) The suspension was homogenized at 0°C under sterile conditions in a Potter-Elvehjem tissue grinder with a small amount of alumina. A microscopic examination of the homogenate revealed a very few intact nuclei. (A series of media, including FTS, which were inoculated with the homogenate remained sterile during 14 days of incubation.)

The total nitrogen of the homogenate was 3.94 mg/ml as determined by Lang's method (*4*). The homogenate was analyzed for nucleic acid according to the modified Schmidt-Thannhauser procedure (*5*), with the use of salmon sperm deoxyribonucleic acid (DNA) and *d*-xylose as standards. The homogenate contained 76 μg of DNA (as DNA) and 780 μg of ribonucleic acid (as pentose) per milliliter.

All experimental and control inoculations involved 6-to-8-week-old mice of the C57 B1/6 strain. The cultures which were to be tested on mice were grown routinely for 48 hours in tubes containing 10 ml of FTS. In experiments I and II, as well as in control experiment B, 0.5-ml aliquots of trichomonad suspensions were inoculated into each tube.

In experiment I, (i) a 48-hour culture of YGA strain maintained for three transfers on CPLMNA was centrifuged, and the centrifugate was washed twice with Earle's balanced salt solution containing penicillin and dihydrostreptomycin. (ii) The final centrifugate was resuspended in 5 ml of 0.137*M* sodium chloride and, upon addition of 1 ml of the JB homogenate, was incubated at 37°C for 8 hours. (iii) Following incubation, 0.5-ml aliquots of the suspension were inoculated into tubes, each containing 10 ml of CPLMNA. The organisms were carried through two transfers on this medium. (iv) A 48-hour culture from the second transfer was treated as in (ii) and (iii) above, except that 1.5 ml of JB homogenate was added to the suspension of trichomonads. (v) After 8 hours' incubation, the suspension was grown on FTS. Mice were inoculated with trichomonad cultures from this medium.

In experiment II, the contents of seven large lesions from experiment I were suspended in FTS containing penicillin and dihydrostreptomycin. Appropriate aliquots of the suspension were inoculated into FTS, which provided the material for injections into mice.

In control experiment A, mice were inoculated with 48-hour cultures of untreated YGA strain. In control experiment B, YGA cells were treated as in steps (i) to (iii) of experiment I, except that 8 μg of crystalline deoxyribonuclease (Sigma Chemical Co.) were added to the suspension containing the parasites and the JB homogenate. After 8 hours' incubation, 0.5-ml aliquots of the suspension were inoculated into FTS. As in experiments I and II, the mice were injected with 48-hour cultures grown on this medium.

All control and experimental lesions were routinely spot checked for sterility. No foreign microorganisms were found on any media, but invariably excellent trichomonad cultures were obtained on FT and FTS.

All experimental data are summarized