Crystalline Human Urokinase: Some Properties

Abstract. *Human urokinase, an enzyme which activates plasminogen, has been crystallized. Repeated recrystallization yielded preparations of maximum specific activity; such preparations revealed only one component when subjected to polyacrylamide gel disc electrophoresis. Studies with the ultracentrifuge demonstrated that urokinase undergoes a concentration-dependent reversible association at* p*H 6.8. When the partial specific volume of the enzyme is assumed to be 0.735 milliliters per gram at 10°C, Archibald measurements indicate the molecular weight of urokinase to be 53,000.*

The enzyme urokinase, which occurs in trace quantities in human urine, catalyzes the conversion of the enzymically inactive precursor, plasminogen, into the active proteolytic enzyme plasmin. Among the diverse substrates susceptible to the lytic action of plasmin is fibrin, the matrix of blood clots. Because plasminogen occurs in blood clots and in blood plasma, the possibility that the plasminogen-activating property of urokinase may have therapeutic utility is widely recognized (*1*). Specifically, attention is being directed to the potential usefulness of urokinase as a thrombolytic (fibrinolytic) agent in acute episodes of thromboembolic disease (for example, coronary thrombosis) and in other conditions associated with a pathological fibrin deposition.

Several of the more fundamental chemical and biological studies of urokinase have been hindered by the lack of homogeneous preparations. Our investigations were undertaken in an effort to resolve this problem.

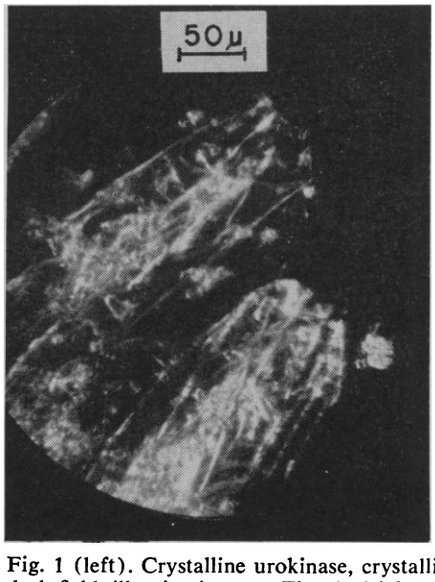
Since the discovery of urokinase more than a decade ago (*2*), a number of procedures for the (partial) purification of the enzyme have been described (*3*). Recently, by starting with aqueous solutions of highly purified preparations of human urokinase (*4*), we have succeeded in obtaining urokinase in crystalline form (Fig. 1). The preparations from which crystals were obtained were those which exhibited a specific activity equal to, or greater than, 40,000 CTA units (*5*) per milligram of protein (*6*) and which revealed no more than three components when subjected to polyacrylamide gel disc electrophoresis (*7*). Crystallization was induced at *p*H 5.0 to 5.3 and at a temperature of 4°C by the cautious addition of sodium chloride with gentle stirring until the urokinase solution assumed a faintly turbid appearance; shortly thereafter, a "silky" sheen became evident on stirring. The sodium chloride concentration was then increased gradually over a period of several days until 98-percent saturation was attained. The urokinase crystals were colorless, thin plates of unusual fragility and brittleness; under the microscope, they were easily shattered into fragments by the application of only moderate pressure to the cover slip.

The once-crystallized preparations were recrystallized until the specific activity reached a constant maximum value; two or three recrystallizations were generally required. A mean loss of 12 percent of the total activity was sustained with each recrystallization. The maximum specific activity attained with three lots of crystals was 652,000 ± 29,800 (standard deviation) CTA units per milligram of nitrogen (Kjeldahl), or 104,000 ± 4,800 CTA units per milligram of estimated protein (*6*). The rise of the specific activity to a constant value on repeated recrystallization indicated that the crystals were indeed urokinase. Twenty-nine milligrams were obtained from 2300 liters of urine from human males, representing an overall yield of 24 percent. In the subsequent sections of this paper, such recrystallized products will be designated "crystalline urokinase."

Solutions of crystalline urokinase, when subjected to polyacrylamide gel disc electrophoresis (*7*), revealed only one component (Fig. 2). At the stage immediately prior to the initial crystallization, two major components and occasionally a third (minor) component could be detected. In these studies, the degree of resolution obtained by the disc-electrophoretic analytical technique surpassed that obtained with other zonal (paper, cellulose acetate, starch gel) electrophoretic procedures.

The crystalline urokinase preparations were also examined for homogeneity in the ultracentrifuge (*8*). A single, symmetrical sedimentation boundary was observed during the ultracentrifugation of solutions of crystalline urokinase at either *p*H 2.1 or 6.8 (*9*), but at the latter *p*H value, boundary spreading was relatively excessive. These observations suggested that, at *p*H 6.8, urokinase may be undergoing rapidly equilibrating association-dissociation reactions (*10*).

That urokinase aggregates reversibly at *p*H 6.8 was established by determining the concentration-dependence of the weight-average molecular weight of the crystalline enzyme in solutions at this *p*H value. In these studies, molecular weights were determined at the menisci by the Archibald method (*8*, *11*) with the assumption that the partial specific volume of urokinase is 0.735 ml/g at 10°C. Concentrations at the menisci were calculated by the equation of Klainer and Kegeles (*12*)



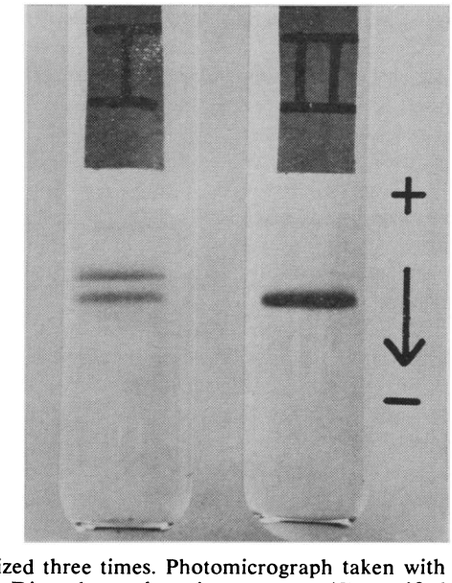


Fig. 1 (left). Crystalline urokinase, crystallized three times. Photomicrograph taken with dark-field illumination. Fig. 2 (right). Disc electrophoresis patterns: (*I*) purified urokinase fraction used as starting material in the crystallization procedure (50 μg of protein); (*II*) crystalline urokinase (100 μg of protein).

and experimental measurements were made in accordance with Schachman's procedure (*13*).

The molecular weight data obtained at *p*H 6.8 over a range of meniscus concentrations of urokinase are presented graphically in Fig. 3. It is evident that the reciprocal of the weight-average molecular weight is linearly related to concentration over the range of concentrations studied. Least-square treatment of these data yielded the following equation for the regression line:

$$\frac{10^5}{M_w} = 1.853 - 0.0864(c)$$

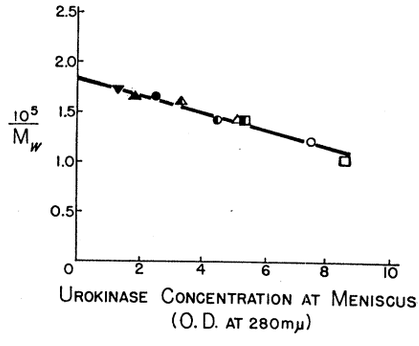


Fig. 3. The concentration-dependence of the weight-average molecular weight of urokinase at *p*H 6.8. The optical density (OD) values pertain to a light path 1.00 cm in length. Ultracentrifugation was performed at 10.0°C in 0.21*M* NaCl, 0.07*M* phosphate buffer at *p*H 6.8 in double-sector, synthetic boundary cells of the capillary type; the protein concentrations at the air-liquid meniscus and the molecular weight values were calculated as defined in the text. The solutions at each of the initial concentrations employed were centrifuged at each of three different rotor speeds in order to achieve differing degrees of protein depletion at the air-liquid meniscus; measurements were made 60 minutes after the respective rotor speed had been attained. The initial concentration (c_0) of each solution was determined after the series of Archibald measurements at three rotor speeds had been completed; the solution recovered from the c_0 determination was then utilized for the series of Archibald analyses at the next lower initial concentration. In the figure above, each symbol is used to define both the initial urokinase concentration (c_0) and the rotor speed at which the respective Archibald measurements were made: open symbols represent c_0 equivalent to $OD_{280} = 11.12$; half-closed symbols represent c_0 equivalent to $OD_{280} = 6.34$; closed symbols represent c_0 equivalent to $OD_{280} = 3.35$; square symbols designate a rotor speed of 8,225 rev/min; circular symbols designate a rotor speed of 11,272 rev/min; upright triangular symbols (for example, △) designate a rotor speed of 16,200 rev/min; the inverted triangular symbol (▼) designates a rotor speed of 20,410 rev/min.

in which M_w is molecular weight and c is the urokinase concentration expressed in terms of the optical density at 280 mμ in 1.00-cm cells (*14*). At infinite dilution, one calculates a value of 54,000 ± 900 (standard error) for the molecular weight of urokinase; this value is, of course, predicated on the assumption that the partial specific volume is 0.735 ml/g at 10°C.

Molecular weight studies similar to those performed at *p*H 6.8 were also performed on urokinase solutions at *p*H 2.1 and at *p*H 10.5 (*15*). Within the limits of experimental precision, no concentration-dependence of the apparent molecular weight was observed at either *p*H 2.1 or 10.5 over the concentration ranges studied. At *p*H 2.1, the mean value and the standard error for a series of eleven determinations of molecular weight were found to be 52,500 ± 530. At *p*H 10.5, the mean value and the standard error for a series of eight determinations were found to be 53,300 ± 440. Each of these values is in good agreement with the value of 54,000 derived by extrapolation of the data at *p*H 6.8 to infinite dilution. Presumably, aggregation is inhibited at the more extreme *p*H values by the repulsive effects of the electrostatic charges on the monomeric molecules; alternatively, relatively minor degrees of aggregation may be masked by analytical insensitivity or by a nonideal behavior of the solutions.

The experimental design of the molecular weight studies at each *p*H value was such that it also provided a further test of the homogeneity of the crystalline urokinase preparations; moreover, at each *p*H value, the design permitted the examination of a single sample over a broad range of meniscus concentrations. During the Archibald determinations, each of several different initial concentrations of urokinase was subjected to three different centrifugal fields (see Fig. 3). In ultracentrifugally polydisperse systems, the higher centrifugal fields cause a preferential depletion of the more rapidly sedimenting molecular species from the region of the meniscus. When the polydispersity is due to rapidly reversible aggregation, such preferential depletion leads to a reequilibration of the molecular species to yield a solution whose composition is identical with that attained by simple dilution to the same solute concentration. The weight-average molecular weight of the solute at the meniscus of such systems, while concentration-dependent, is independent of whether the reduction in concentration is achieved primarily by simple dilution or by ultracentrifugal fractionation. Such was found to be the case with urokinase solutions at *p*H 6.8 (Fig. 3, for example, the region wherein the optical density is approximately 5). At each of the other two *p*H values studied, the homogeneity of crystalline urokinase was further indicated by the lack of any detectable dependence of the Archibald molecular-weight values upon the magnitude of the applied centrifugal fields.

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5. The CTA unit refers to the standard unit of urokinase activity adopted by the Committee on Thrombolytic Agents, National Heart Institute: S. Sherry, N. Alkjaersig, A. P. Fletcher, *J. Lab. Clin. Med.* **64**, 145 (1964).
6. Unless otherwise indicated, protein concentration was calculated to be 6.25 times the nitrogen concentration; nitrogen concentration was determined by the micro-Kjeldahl procedure. A solution of crystalline urokinase containing 0.100 mg of nitrogen per milliliter exhibited an optical density of 0.853 at 280 mμ in 1.00-cm cuvettes.
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8. All ultracentrifuge studies were conducted at 10.0°C in a Spinco model E analytical ultracentrifuge equipped with a phase plate, schlieren optical system, and a rotor temperature indicator and control unit.
9. At each *p*H value the buffer consisted of 0.07*M* phosphate in 0.21*M* NaCl; the urokinase concentration was 0.3 percent; the rotor speed was 59,780 rev/min and the duration of ultracentrifugation was 85 minutes or more.
10. If the rate of adjustment of the association-dissociation equilibrium were not greater than the rate of ultracentrifugal separation of the different molecular species (monomers, dimers, for example), more than one boundary would probably have been evident during sedimentation; H. K. Schachman, *Ultracentrifugation in Biochemistry* (Academic Press, New York,

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14. In this equation, the coefficient of c will, of course, be influenced by any of the factors which affect protein aggregation, for example, ionic strength and temperature.
15. At *p*H 2.1 (0.21*M* NaCl, 0.07*M* phosphate buffer), the investigated range of meniscus concentrations corresponded to an optical density range of 2.2 to 11.4 at 280 mμ in cells 1.00 cm in length. At *p*H 10.5 (0.21*M* NaCl, 0.07*M* glycinate buffer), the investigated range of meniscus concentrations corresponded to an optical density range of 2.1 to 11.6 at 280 mμ in cells 1.00 cm in length. At each *p*H value, the respective ranges of meniscus concentrations were attained by the procedure described in the text accompanying Fig. 3; the respective series of rotor speeds used in each study were identical.

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Neurosecretory Processes Extending into Third Ventricle: Secretory or Sensory?

Abstract. *"Dendrites" projecting into the third ventricle from hypothalamic neurosecretory neurons of the Pacific tree frog,* Hyla regilla, *appear to be morphologically equipped to serve both secretory and sensory functions. These cell processes contain elementary neurosecretory granules, Golgi membranes, mitochondria, and endoplasmic reticulum, suggesting synthesis and possibly release of hormonal material. Each "dendrite" is provided with a single cilium which has an accessory centriole and a striated rootlet.*

"Dendrites" extending from hypothalamic neurosecretory cells toward the lumen of the third ventricle have been described repeatedly in various vertebrates by light microscopists (*1, 2*). Stainable neurosecretory material in such processes often appears to project into the ventricle between ependymal cells, suggesting that these neurons may release their hormonal products into the cerebrospinal fluid as well as into the blood stream in the neurohypophysis (*2*). Dierickx has proposed instead that the "dendrites" act as receptors responding to osmotic changes in the cerebrospinal fluid (*3*).

In the course of an ontogenetic study of the neurosecretory system in the Pacific tree frog, *Hyla regilla*, the "dendrites" of adult preoptic neurons were observed with the electron microscope. The processes appear to be particularly prominent in this species (*2*) (Fig. 1), occurring as stout continuations of the perikaryon and usually ending in bulbous expansions at the luminal surface of the ventricle (Figs. 2 and 3). There is variation in the shape of the endings, however; some are not expanded, whereas others appear to spread over the surface of adjacent ependymal cells. The smooth surfaces of the processes contrast with the irregular outlines of ependymal cells which have numerous microvilli at their free surfaces and extensive interdigitations at the lateral and basal borders (Figs. 2 and 3).

All the organelles of the neurosecretory perikaryon, including endoplasmic reticulum, ribosomes, mitochondria, Golgi membranes, and elementary neurosecretory granules, are found throughout the process (Figs. 2 and 3). Typical terminal bars and desmosomes occur between the processes and adjacent ependymal cells. Extending into the lumen from the tip of the process is a single cilium provided with a striated rootlet (Figs. 3 and 4) and an accessory centriole (Fig. 4). The major branch of the rootlet has been followed to a depth of over 5 μ into the process. Occasionally, tenuous branches of the rootlet are also seen (Fig. 4). It has not been determined whether these cilia have the two central fibrils characteristic of kinocilia (*4*).

The finding of elementary granules in the processes may indicate secretory activity or merely that synthesis occurs here as well as in the cell body proper. Inasmuch as all of the structural components normally associated with synthesis are found in the processes, granules could be manufactured in the "dendrites" and then could move centripetally to the perikaryon and down the axon to be released in the neurohypophysis. On the other hand, although no intact granules were observed in the lumen or passing through the plasma membrane, release from the "dendrite" into the ventricle could occur by diffusion, as is apparently the case in the axon endings of these cells in the neurohypophysis. Furthermore, an apocrine type of secretion remains a distinct possibility. It is postulated that the granules and droplets described by light microscopists as representing secretion into the ventricle lumen (*2, 5*) are really portions of the cytoplasm of the processes, containing secretory granules and thus staining with paraldehyde fuchsin. In many instances, the light microscope would not reveal a connection to the cell body because of the low concentration of secretory granules in the proximal part of the process. In other instances, cytoplasmic fragments, even under the electron microscope, appear to be disconnected from the surface; serial sections are required to establish whether these are in fact free in the lumen.

The whole question of secretion into the ventricle is difficult to assess, for there is a surprising lack of data on the content of neurohypophysial hormones in the cerebrospinal fluid (*6*), considering the numerous proposals of secretion into the ventricle, either from "dendrites" (*1, 2, 5*) or from axon endings in the neurohypophysis (*7*).

Dierickx's suggestion that the dendrites serve as osmoreceptors has some cogency, and, as he pointed out, this function could coexist with secretion into the ventricle (although this is unlikely if secretion is of the apocrine type). Unfortunately, the small amount of evidence is inferential. It is known that osmoreceptors exist somewhere within the vertebrate body, and perfusion experiments indicate location in the brain itself (*8*). Because hypothalamic neurosecretory cells respond to osmotic manipulation by changes in content of hormone and of stainable secretory material (*9*), the "dendrites" are considered the most likely candidates as such receptors.

Presence of a cilium at the tip of the process is certainly compatible with a sensory function, as many known receptors are associated with a ciliary structure (*4*). However, survey of the literature shows that no specific modification is invariable in sensory cilia: they may or may not have two central fibrils; they may or may not have accessory centrioles; they may or may not show structural modifications of the distal portion of the cilium (for example, *4, 10*). In addition, cells not known to be sensory may have cilia lacking central fibrils and also may have accessory centrioles (*11*). Thus, observed morphologic features of neurosecretory "dendrites" leave their possible sensory function unresolved.

It is conceivable that the preoptic neurosecretory cells are derived from ependyma (*12*) and that therefore the "dendrites" merely remain attached to ependymal cells when the neurosecretory cell bodies proper sink into the underlying brain tissue. Further studies