that of pure dimethylsulfide (Fig. 1). Mass spectrography provided further evidence.

Analysis was performed on 200 ml of cat breath collected over a 5-minute period shortly after the intravenous injection of 400 mg of the sulfoxide per kilogram of body weight, which is approximately the maximum tolerated single dose. Figure 2 shows that pure dimethylsulfide appears at mass 62. The peak at 35 is quite rare and characteristic of the sulfide. The fragment patterns for the breath sample (Fig. 2*A*) and pure dimethylsulfide (Fig. 2*B*) are identical. The sulfide was absent in a sample of the injected dimethylsulfoxide analyzed by both gas chromatographic and mass spectrographic techniques.

These studies are highly suggestive of the existence of a metabolic pathway in the cat which reduces the sulfoxide to the sulfide. Mazel *et al.* (*6*) have reported that in contrast to some other *S*-methyl compounds, dimethylsulfide is not *S*-demethylated by rat liver microsomes. Although their studies were carried out in vitro, their results lend support to the contention that dimethylsulfide is a terminal metabolite of dimethylsulfoxide. This does not exclude the existence of other metabolic pathways.

Also noted in the course of these studies was the appearance of a deep red coloration of the urine. Preliminary spectrographic analysis of bladder urine from cats treated with dimethylsulfoxide indicates the presence of both hemoglobin and methemoglobin.

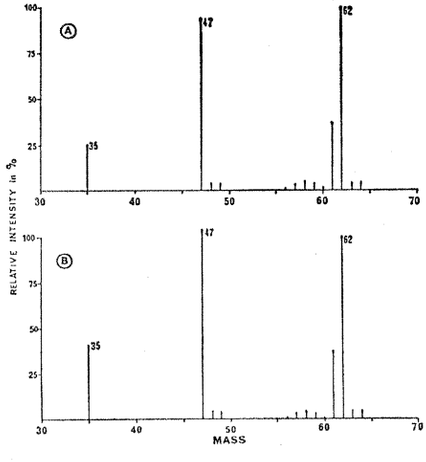

Fig. 2. Mass spectrograms of cat's exhaled breath (*A*) and dimethylsulfide (*B*) (*8*). The sample of exhaled air was exhausted through a liquid nitrogen–chilled trap. The contents of the trap were admitted to the mass spectrometer by warming. *B*, dimethylsulfide introduced by air exhaustion.

VICTOR DISTEFANO
HAROLD H. BORGSTEDT

Department of Pharmacology, School of Medicine and Dentistry, University of Rochester, Rochester, New York

References and Notes

1. M. J. Ashwood-Smith, *Nature* **190**, 1204 (1961).
2. H. B. Banner and R. M. McCormack, *Surg. Forum* **13**, 478 (1962); C. Huggins, *ibid.* p. 51.
3. S. W. Jacob, M. Bischel, R. D. Herschler, *Current Therap. Res.* **6**, 134 (1964).
4. T. L. Sourkes and Y. Trano, *Arch. Biochem. Biophys.* **42**, 321 (1953); S. Black, E. M. Harte, B. Hudson, L. Wartofsky, *J. Biol. Chem.* **235**, 2910 (1960).
5. M. A. Bennett, *Biochem. J.* **33**, 1794 (1939).
6. P. Mazel, J. F. Henderson, J. Axelrod, *J. Pharmacol. Exptl. Therap.* **143**, 1 (1964).
7. Anesthesia: Dial-urethane solution (Ciba), 0.63 ml/kg. Microtek 2500R gas chromatograph; direct injection of liquid samples. Columns: 1 m × 6 mm, 20 percent glycerol on Ultraport; then 3.5 m × 6 mm of diisodecyl phthalate on Ultraport at 85°C. Carrier: helium at 2.4 atm inlet pressure and 100 ml/min flow. Thermal conductivity detector at 150°C and detector current of 640 ma.
8. Anesthesia: Dial-urethane solution (Ciba), 0.63 ml/kg. Magnetically scanned, motor-driven mass spectrometer of the 60° sector type. Peaks normalized to mass 62 = 100 percent in both records.

9. We thank G. P. Happ and David W. Stewart of the Research Laboratories, Eastman Kodak Co., for the mass spectrographic analysis. Supported in part by the U.S. Atomic Energy Commission under contract AT(30-1)-2192.

26 February 1964

Linear Polymerization of a Gastropod Hemocyanin

Abstract. *The decreased solubility of a gastropod hemocyanin in the region of the isoelectric point has been correlated with a sharp increase in light scattering produced by the protein in solution. Electron microscopic observations on negatively stained spray preparations in the isoelectric region demonstrated that the formation of linear polymers of the molecule underlies both the increase in light scattering as well as the decreased solubility of the protein. While the linear polymerization produces chains of varying length with no evidence of interaction between chains, the polymerization proceeds with a high degree of ordering of individual molecules. On either side of the isoelectric region of this hemocyanin two forms of molecules are present, rectangular and circular. The rectangular molecules polymerize in an end-to-end fashion in the isoelectric region. The rectangular molecules which participate in the polymerization appear to be divided perpendicularly to their length into three major segments.*

Hemocyanin, the copper-containing respiratory protein of various invertebrate phyla, was among the first proteins to be characterized by ultracentrifugation (*1*). These and subsequent studies led to the first accurate determination of molecular weights for such macromolecules (*2*). Among these fundamental observations was the demonstration of dissociation of hemocyanin on either side of the isoelectric point (*2*) into particles whose weights are characteristically halves or eighths of that of the original associated molecule (*3*). These changes may be observed visually; for example, the hemocyanin of *Kelletia kelletia* (the sea snail) in associated form is an opalescent gray-blue exhibiting a marked Tyndall effect, but at the *p*H where the dissociation occurs and above that *p*H, it is a clear dark blue.

It has not yet been possible to reconcile the data on size and shape of those proteins in solution from sedimentation and diffusion studies with the appearance of dried hemocyanin in the electron microscope (*4*). Brohult (*5*) concluded, on the basis of the high frictional coefficients, that the hemocyanin of *Helix pomatia* existed in solution as highly asymmetrical particles. Assuming their shape to be elongated prolate ellipsoids, he calculated their length to be 1130 Å and their width to be 136 Å. Dissociation involved splitting in a plane parallel to the long axis and resulted in two half molecules with lengths of 890 Å and widths of 60 Å. Electron micros-

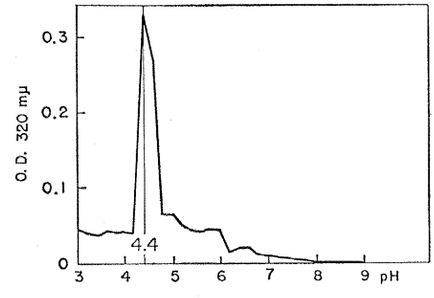

Fig. 1. Light scattering by *Kelletia kelletia* hemocyanin, 100 μg/ml, *p*H 2.1 to 6.0, 0.02*M* acetate buffer; *p*H 6.0 to 9.1, 0.02*M* phosphate buffer; *p*H increments 0.2 units; optical density at 320 mμ.

copy of negatively stained spray preparations of *Helix pomatia* hemocyanin showed rectangular and circular molecules with lengths of 335 Å, widths of 300 Å, and diameters of 300 Å (*6*). These findings were reproducible and there was no evidence of particles or chains of particles (*4*) 1130 Å in length and 136 Å in width. A single cylindrical form for the hemocyanin molecule was proposed: the rectangular and circular forms were merely two different views of the same molecule (*7*).

In our laboratories, we have been concerned with the shape and size of proteins of high antigenicity, among them the hemocyanins. A recent focus of study has been the nature of the dissociation and aggregation phenomena of the hemocyanin of the sea snail *Kelletia kelletia* (*8*). It is prepared from the animal's hemolymph by three cycles of centrifugation consisting of sedimentation at 78,000*g* for 3 hours, resuspension, and precipitation of debris and other aggregates at 5000*g* for 1 hour. Immunoelectrophoresis, using rabbit antibody to hemocyanin, showed a single broad migrating component made up of two lines of hemocyanin antibody-antigen precipitates. The two bands may indicate two antigenic determinants or possibly two different molecular species.

While the isoelectric point as classically defined is the *p*H of a buffer of specified composition in which no net migration of the protein is produced by application of an electric field, for many proteins it also represents the region of least solubility (*9*). The isoelectric region of *Kelletia kelletia* hemocyanin was determined by measuring the light scattering over a range of *p*H's. The marked increase in light scattering over a narrow *p*H range was used as an approximation of the isoelectric point. The purified protein was suspended in 0.02*M* acetate buffer in a concentration of 100 μg/ml, and the *p*H was varied from 2.1 to 9.1 in 0.2 *p*H unit increments. The solutions were read in a Beckman DU spectrophotometer at 320 mμ. Figure 1 shows the result of these studies. There is a sharp increase in optical density at *p*H 4.2 which reaches a peak at *p*H 4.4. When the *Kelletia kelletia* hemocyanin at this *p*H, ionic strength, and concentration is allowed to stand, it precipitates from the solution.

The problem, which has been approached by electron microscopy, is whether this gross aggregation and precipitation phenomenon is random or whether it represents a specific organization of molecules. The electron microscopic studies were made of spray preparations of hemocyanin at a concentration of 500 μg/ml in 0.02*M* ammonium acetate volatile buffer. Two-percent uranyl acetate was added immediately before spraying on carbon-coated grids. Preparations of the aggregated form (at

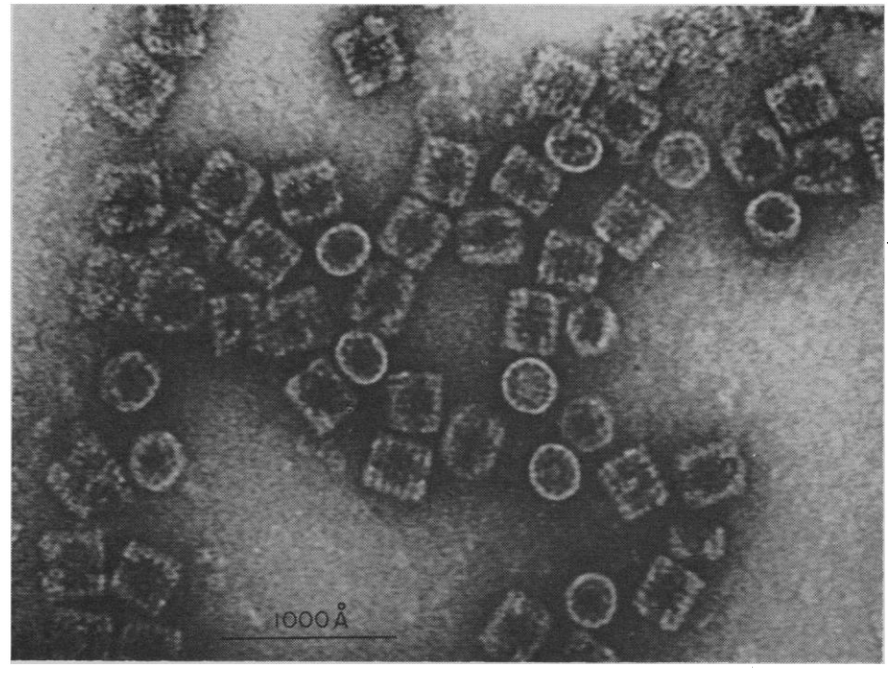

Fig. 2. *Kelletia kelletia* hemocyanin, *p*H 3.4, 500 μg/ml, negatively stained with 2 percent uranyl acetate, 0.02*M* ammonium acetate buffer. The circular molecules are 310 Å diameter, and the rectangular molecules are 330 Å long and 290 Å wide. Note the three major divisions perpendicular to length.

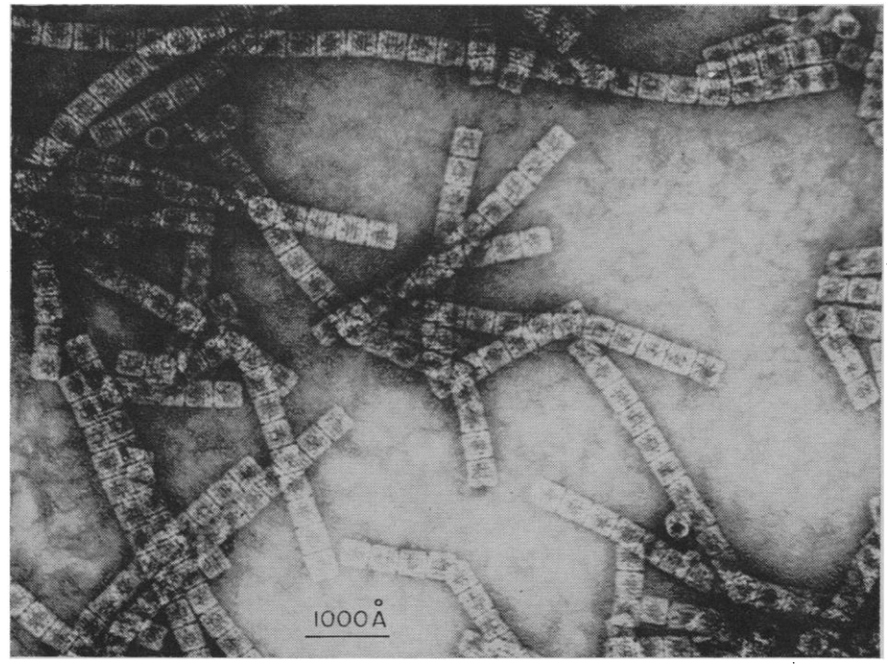

Fig. 3. Linear polymers of *Kelletia kelletia* hemocyanin in region of isoelectric point, *p*H 4.8, 500 μg/ml, negatively stained with 2 percent uranyl acetate 0.02*M* ammonium acetate buffer. Polymer length is variable, width 355 Å. Polymers consist of rectangular molecules oriented end to end. Few circular molecules are present.

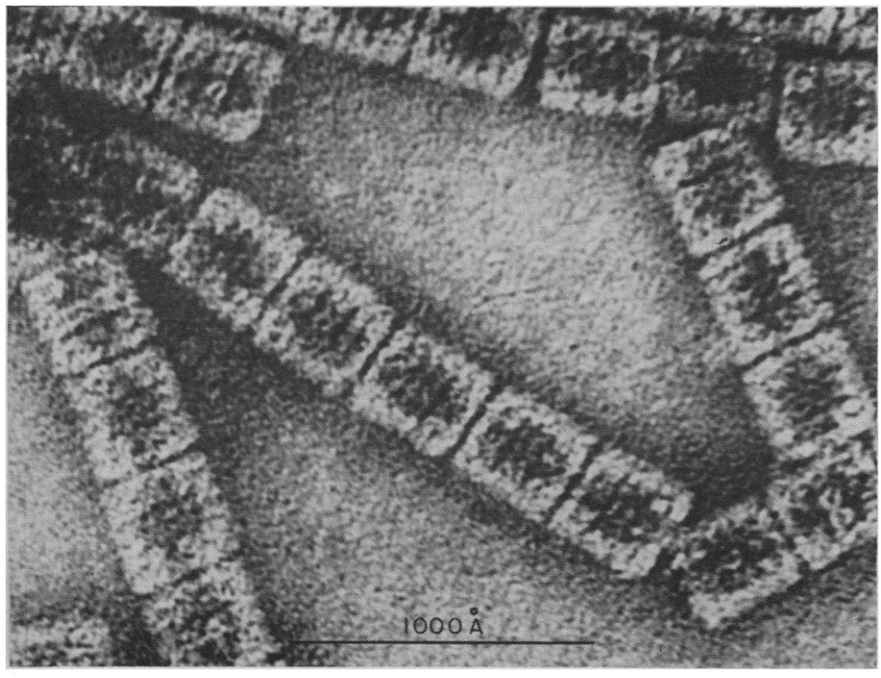

Fig. 4. Monomers of the linear polymer. Conditions of preparation same as in Fig. 3. Individual monomers making up the polymers have length 365 Å, width 355 Å. Each molecule appears divided perpendicular to length into three major segments. Each segment suggests two helical coils, 60 Å in width and 355 Å in length.

the isoelectric point) and the nonaggregated form (confirmed by light scattering and sedimentation analysis at *p*H 3.4) were viewed in the Siemens Elmiskop I electron microscope (*10*) at instrument magnifications of 40,000 and 80,000. Figure 2 shows two types of molecules of sea snail hemocyanin at *p*H 3.4. The rectangular molecules have widths of 290 Å and lengths of 330 Å. A segmentation perpendicular to the length dividing the forms into three or six equal segments is apparent. The circular molecules have diameters of 310 Å and show internal structure. The forms and overall dimensions of these preparations agree very closely with those reported recently for *Helix pomatia* hemocyanin (*6, 7, 11*). The studies on sedimentation rates and *p*H dissociation indicate that *Kelletia kelletia* and *Helix pomatia* hemocyanins have similar molecular weight and *p*H dissociation (*12*). Extensive studies, which will be reported later, suggest that, for *Kelletia kelletia* hemocyanin, the circular and rectangular forms represent two different species of molecules.

The negatively stained spray preparation of *Kelletia kelletia* hemocyanin in the isoelectric region shows a remarkably high degree of molecular organization (Fig. 3). The characteristic forms are linear polymers of varying length representing end-to-end aggregations of the hemocyanin particles. The orientation of the polymers seems to be random, without evidence of interaction between polymers. Figure 4 shows the polymers at high magnification: the internal segmentation characteristic of the rectangular forms can be distinguished, suggesting that the association and orientation of the molecules at the isoelectric point involves the 355-Å ends of the rectangles. Under the conditions of preparation, no characteristic dimension other than width (355 Å) is evident, the polymers varying in length.

That the *p*H range of maximal polymerization in electron microscopic preparations (4.8) is not identical to the *p*H of the optical-density peak (4.4) may be a reflection of changes in *p*H occurring during the drying phase of the negative staining process. This is consistent with the observation of Bradley (*13*) that uranyl acetate buffer in this *p*H range underwent elevation in *p*H in the drying process. Examination of preparations over a wide *p*H range indicated that the polymerization occurred only in this narrow *p*H range.

Concerning the nature and type of forces operating in producing the high degree of molecular organization, one factor may possibly be the conditions of preparation of this protein for final viewing in the electron microscope which involves a drying in a vacuum. Since hemocyanin is no longer soluble in water after lyophilization (*12, 14*), it is likely that the specimens prepared for electron microscopy are also modified to a great extent. The high degree of structural organization and the reproducibility of these forms suggest that these molecules as observed in the electron microscope are a direct result of the amino acid composition and sequence, and therefore represent the configuration of the molecule under the conditions of preparation. Thus, we assume that we are seeing the hemocyanin molecule in its most stable form but under very unfavorable conditions.

The affinity of the ends of each molecule in the chain with its neighbors might indicate that the forces operating in maintaining the size and shape of the rectangular molecule at *p*H's on either side of the isoelectric point are similar. Studies have been done on the mechanism of dissociation and reassociation on hemocyanins of this and other species (*15*).

RICHARD M. CONDIE
RICHARD B. LANGER
Department of Pediatrics,
Variety Club Heart Hospital,
University of Minnesota Hospitals,
Minneapolis 14

References and Notes

1. T. Svedberg and E. Chirnoaga, *J. Am. Chem. Soc.* **50**, 1399 (1928).
2. I. Eriksson-Quensel and T. Svedberg, *Biol. Bull.* **71**, 498 (1936).
3. S. Brohult and S. Claesson, *Nature* **144**, 111 (1939).
4. I. Claesson, *Arkiv Kemi* **10**, 1 (1956).
5. S. Brohult, *J. Phys. Colloid Chem.* **51**, 206 (1947).
6. E. F. J. van Bruggen, E. H. Wiebenga, M. Gruber, *Biochim. Biophys. Acta* **42**, 171 (1960).
7. ———, *J. Mol. Biol.* **4**, 1 (1962).
8. Obtained from Dr. Rimmon Fay, Pacific Bio-marine, Santa Monica, California.
9. R. A. Albert, in *The Proteins*, H. Neurath and K. Bailey, Eds. (Academic Press, New York, 1953), vol. 1, part A, p. 478.
10. We thank Dr. J. Spizizen, Department of Microbiology, University of Minnesota, for cooperation in providing electron-microscopic facilities.
11. O. Levin, *Acta Univ. Upsal.* **24**, 1 (1963).
12. Unpublished observations of R. M. Condie.
13. D. E. Bradley, *J. Gen. Microbiol.* **29**, 503 (1962).
14. M. Litt and W. C. Boyd, *Nature* **181**, 1075 (1958).
15. R. B. Langer and R. M. Condie, *J. Cell Biol.* **19**, 43A (1963).
16. Aided by grants from the USPHS (NB-02042 and 5TI-HE-5462), the National Foundation, Clinical Research Center (H-6314) and the American Heart Association. We thank Katherine Pih and Carolyn Hakim for technical assistance and Professor R. A. Good for support and encouragement.

10 February 1964