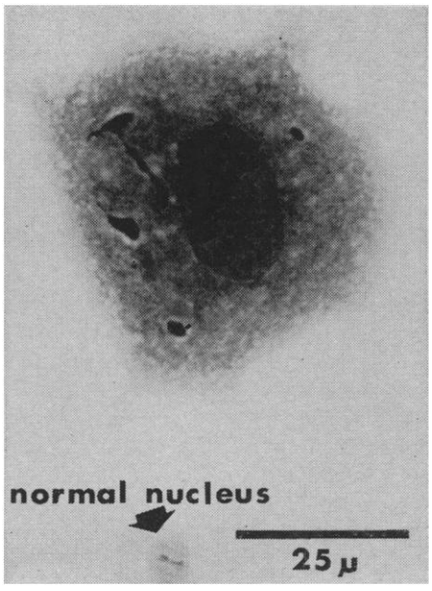

Fig. 3. Mature, 24-day-old, unencapsulated lymphocystis cell produced in vitro and stained with May-Grünwald-Giemsa. Encapsulation normally obscures internal detail: therefore, a nonencapsulated cell was selected for illustration.

cells are mature at 3 to 4 weeks and have the identical appearance of natural lymphocystis cells in fin webs of perch (*Acerina cernua*) (*9*) (Fig. 2). Lymphocystis cells that develop from centrarchid cells in vitro may reach a length of several hundred microns before becoming encapsulated but afterward they are smaller than those which occur in the fish. Encapsulated mature cells in the bluegill are usually over 100 μ in greatest length and commonly measure several hundred microns (*10*). In contrast, encapsulated cells in culture seldom exceed 100 μ in length and the largest are usually 40 to 90 μ. Inclusion bodies, the apparent sites of viral replication (*4*, *5*), form extensive fenestrated networks in infected centrarchids. Similar differentiation occurs in some cells in culture (Fig. 3), but in others the inclusions remain dense and homogeneous although there usually are several inclusions in each mature cell.

Cell cultures and young susceptible fish have comparable sensitivity to lymphocystis virus. Homogenates of mature lesions contained about 10^7 ID_{50}/ml (infective dose, 50 percent effective) when assayed in young bluegills (*4*, *8*). Infectivity of similar material in bluegill cell cultures was determined at both laboratories and found to be about $10^{6.5}$ ID_{50}/ml. Though not intended as a comparison, infected cell cultures (tubes, 16 by 125 mm, seeded with 1.0 or 1.5 ml cell suspension) at maturity generally had a titer of $10^{4.5}$ to 10^5 ID_{50}, most but not all of which was present in the cell sheet.

During the long incubation necessary to develop mature lymphocystis cells, *p*H of the culture medium dropped from an initial 7.6 or 7.8 to 7.0 or lower. Changes of medium were not required, but when the medium was renewed, young lymphocystis cells appeared, generally in "colonies," and this was interpreted as infection by first-generation virus.

Several methods were used successfully to prepare virus suspensions for transfer. Cell sheets were covered with water, exposed to three cycles of freezing and thawing, or simply scraped from culture vessels, and homogenized in tissue grinders.

At LU, 6th-passage virus in cell culture (10^{-15} dilution from original) was homogenized and inoculated into bluegills and into bluegill cell cultures. The titer in cell culture was $10^{6.5}$ ID_{50}/ml, and lymphocystis lesions were grossly evident in fish inoculated with the lower dilutions of virus. Lymphocystis in fish was confirmed by histological examination, and virus was reisolated in cell culture. Cell homogenates from uninfected control cultures produced no lesions in fish.

At EFDL, 9th passage of the virus in cell culture (10^{-20} dilution from original) was similarly used to infect young bluegills. Lymphocystis cell culture medium (0.05 ml) was injected into young bluegills and all developed lesions; histologic confirmation was obtained and virus was reisolated. Fish inoculated with control-culture homogenates did not develop lesions.

Cell-culture homogenates were tested for hemagglutination at *p*H 7.2 to 7.3, 4° and 20°C. Washed cells from the following animals were tested: bluegill, largemouth bass, bullfrog (*Rana catesbeiana*), painted turtle (*Chrysemys picta*), chicken (*Gallus domesticus*), rabbit (*Oryctolagus cuniculus*), guinea pig (*Cavia porcellus*), sheep (*Ovis musimon*), and man (*Homo sapiens*) (O+). With the possible exception of material diluted no more than 1:2 with frog and turtle cells at 4°C, lymphocystis virus did not hemagglutinate, neither was there hemadsorption of any erythrocytes by infected cultures at 20°C. Newcastle disease virus, used as a positive control, agglutinated fish, frog, turtle, chicken, and human O+ cells.

When the method of Marmur was used (*11*) spectacular yields of DNA were extracted from lymphocystis lesions in fish. DNA was also extracted by the cold phenol method of Ito (*12*). Neither extract showed infectivity in cell cultures that were susceptible to intact virus, and fish were similarly unaffected.

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References and Notes

1. R. Weissenberg, *Ann. N.Y. Acad. Sci.* **126**, 362 (1965).
2. L. Grützner, *Zentralbl. Bakteriol. Parasitenk. Abt. I Orig.* **165**, 81 (1956).
3. K. Wolf, *Virology* **18**, 249 (1962).
4. ———, *Develop. Ind. Microbiol.* **5**, 140 (1964); ——— and C. P. Carlson, *Ann. N.Y. Acad. Sci.* **126**, 414 (1965); R. Walker, *ibid.*, p. 386.
5. R. Walker, *Virology* **18**, 503 (1962).
6. ——— and K. Wolf, *Amer. Zool.* **2**, 566 (1962); R. Walker and R. Weissenberg, *Ann. N.Y. Acad. Sci.* **126**, 375 (1965).
7. Virus was in 13th animal passage at the Eastern Fish Disease Laboratory. The 12th passage was deposited with the American Type Culture Collection and has been assigned the accession number VR 342.
8. C. E. Dunbar and K. Wolf, *J. Infect. Dis.*, in press.
9. R. Weissenberg, *Arch. Mikroskop. Anat. Entwickl.* **94**, 55 (1920).
10. R. Weissenberg, *Zoologica* **30**, 169 (1945).
11. J. Marmur, *J. Mol. Biol.* **3**, 208 (1961).
12. Y. Ito, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 387 (1962).
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8 November 1965

Lobster Hemocyanin: Properties of the Minimum Functional Subunit and of Aggregates

Abstract. *Lobster hemocyanin dissociates into a functional subunit of 68,000 to 70,000 molecular weight when Ca^{2+} ions are removed from an alkaline solution of low ionic strength. Succinylation results in a further dissociation into two nonfunctional subunits of approximately 34,000 to 35,000 molecular weight. Amino acid analysis and tryptic peptide patterns indicate that the functional subunit is composed of at least two polypeptide chains which are similar.*

Both the dissociation of hemocyanins into subunits and their equilibrium with oxygen are strongly influenced by divalent cations, *p*H, and ionic strength (*1*). This relation suggests that the degree of dissociation into subunits may be

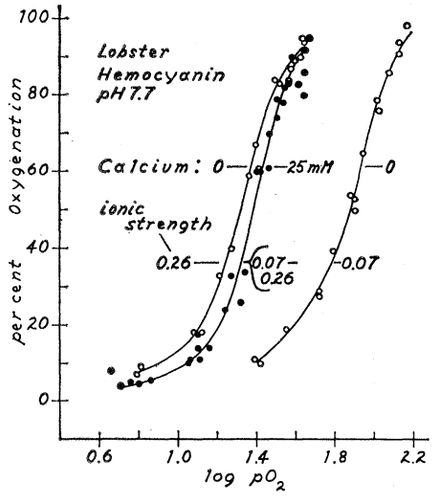

Fig. 1. The oxygen equilibrium of lobster hemocyanin at *p*H 7.7, 20°C, in the presence and absence of Ca^{2+} ions. Protein concentration is approximately 60 mg/ml. Removal of Ca^{2+} from solutions of low ionic strength results in a shift of the oxygen equilibrium curve to the right; in solutions of high ionic strength in the absence of calcium ions a small shift to the left is observed.

an important factor in modifying the oxygen equilibrium. Our experiments show that the removal of calcium from alkaline solutions of low ionic strength results in the dissociation of lobster hemocyanin, first, into half molecules containing six oxygen-binding sites and then into units containing a single site. The first dissociation does not affect the shape of the oxygen equilibrium curve, but dissociation into single-site units is accompanied by complete loss of site-site interactions.

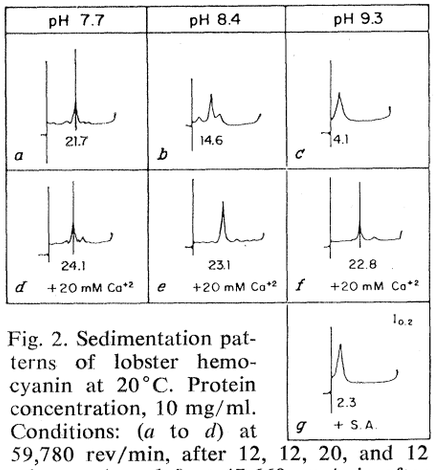

Fig. 2. Sedimentation patterns of lobster hemocyanin at 20°C. Protein concentration, 10 mg/ml. Conditions: (*a* to *d*) at 59,780 rev/min, after 12, 12, 20, and 12 minutes; (*e* and *f*) at 47,660 rev/min after 16 minutes, buffers as described in text; (*g*) succinylated sample in *p*H 9.5 bicarbonate buffer, ionic strength 0.2, at 59,780 rev/min after 48 minutes. *S.A.*, succinic anhydride.

Hemocyanin was obtained by pooling the blood from the pericardia of several New England lobsters *Homarus americanus* (*2*). Solutions intended for oxygenation measurements were dialyzed against 0.05*M* tris of *p*H 7.7 or 8.4 or against 0.05*M* glycine buffer of *p*H 9.3, adjusted to either 0.07 or 0.26 ionic strength by addition of buffer components. Anhydrous $CaCl_2$ (dried at 120°C) was added to certain samples and measured by a colorimetric procedure (*3*). Oxygen equilibrium measurements (*2*) (Fig. 1) show that the addition of Ca^{2+} ions to solutions previously dialyzed against Ca^{2+}-free buffers of low ionic strength results in a threefold increase in oxygen affinity, whereas the addition of Ca^{2+} ions to buffers of high ionic strength results in a very slight decrease in oxygen affinity. Thus at certain values of ionic strength Ca^{2+} ions have no effect. Similar results were obtained at *p*H 8.4 except that the oxygen affinity was higher and the effect of the addition of Ca^{2+} ions was smaller.

Ultracentrifugation (Fig. 2) of the same samples used in Fig. 1 shows that the sedimentation coefficient is 22 to 24*S* in the presence of Ca^{2+} ions. Removal of Ca^{2+} ions by dialysis results in only a slight change at *p*H 7.7 (Fig. 2, *a* and *d*) but the aggregate largely dissociates into half molecules, about 15*S*, at *p*H 8.4 (Fig. 2, *b* and *e*) and dissociates completely into the minimum functional 4*S* unit at *p*H 9.3 (Fig. 2, *c* and *f*). We believe that the 4*S* molecule is the minimum functional unit because two copper atoms are associated with the binding of each oxygen molecule, and the minimum molecular weight corresponding to two copper atoms is about 68,000. A globular protein with this molecular weight has a sedimentation coefficient between about 4.0 to 4.5*S*. A similar minimum molecular weight has been reported for *Limulus polyphemus* hemocyanin (*4*). The data of Fig. 3 show that dissociation into single-site units is accompanied by a large change in the position and shape of the oxygen equilibrium curve. The slope (n) of the line in Fig. 3 indicates absence of interactions between combining sites since $n = 1$. If $n > 1$, facilitating interactions exist (*5*). A similar evaluation for the data at *p*H 7.7 in Fig. 1 shows that $n = 3.6$ when measured at 50-percent oxygenation. The value of n increases with degree of oxygenation and is higher in the presence of Ca^{2+} ions than in their absence. This observation implies that the number of Ca^{2+} ions bound to hemocyanin should be larger in oxy- than in deoxyhemocyanin (*2*). The magnitude of n is a measure of the minimum number of interacting sites. Although the sedimentation data show that the molecule in calcium-free solution splits in half on going from *p*H 7.7 to 8.4, no substantial change occurs in the value of n. This behavior may be similar to that observed with human hemoglobin in solutions of high ionic strength which causes dissociation into half-molecules, yet the value of n does not change (*6*). An association-dissociation equilibrium, molecular relaxation effects, or the absence of true equilibrium among protein tautomers (*7*) have been invoked to explain these phenomena. Our data do not permit choice between these alternatives.

Solid succinic anhydride (45 mg) was added to an aqueous solution containing 90 mg of hemocyanin maintained at *p*H 8 by addition of NaOH (*8*). This treatment results in a further dissociation from a 4*S* to a 2.3*S* unit which is incapable of combining reversibly with oxygen. A typical sedimentation pattern of succinylated hemocyanin is shown in Fig. 2*g*. Succinylated hemocyanin shows only a single major electrophoretic component on starch-gel electrophoresis (*9*) at *p*H 9.5. The 2.3*S* subunit probably corresponds to the 35,000

Table 1. Amino acid analysis of lobster hemocyanin. A molecular weight of 34,350 is assumed. Tryptophane was not analyzed. The values are averages of 24- and 72-hour hydrolyzates except where indicated.

Amino acid	Amino acid residues per molecule protein	
	Average	Integral
Lysine	14.7	15
Histidine	23.3	23
Arginine	14.6	15
Cysteic acid	1.8*	2
Aspartic acid	40.7	41
Threonine	17.3†	17
Serine	16.3†	16
Glutamic acid	33.3	33
Proline	14.2	14
Glycine	17.8	18
Alanine	18.8	19
Valine	19.7‡	20
Methionine	5.9	6
Isoleucine	14.6	15
Leucine	22.4	22
Tyrosine	11.7	12
Phenylalanine	17.8	18

* Obtained by performic acid oxidation (*15*). † Obtained by extrapolation to zero time. ‡ 72-hour value only.

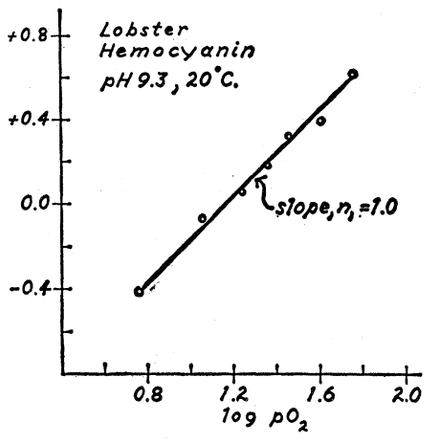

Fig. 3. Oxygen equilibrium data obtained in a Ca^{2+}-free glycine buffer of *p*H 9.3 and ionic strength 0.07, at 20°C. Ordinate is $\log[y/(1-y)]$; y is the fractional degree of oxygenation, and pO_2 is the oxygen pressure.

molecular weight unit seen by Levin (*1*) in electron micrographs of material prepared at *p*H 10.2. These observations indicate that the smallest functional molecule is composed of two subunits.

In amino acid analysis of lobster hemocyanin by standard procedures (*10*) (Table 1), approximately 15 residues each of lysine and arginine were found in a unit having a molecular weight of 34,000. Almost identical analyses were reported for the hemocyanin of the very similar species, *Homarus vulgaris* (*11*). Tryptic peptides from lobster hemocyanin have been separated by automatic column chromatography procedures (*12*). A column (0.9 by 15 cm) of Spinco type 50A resin was used at 50°C with a 250-ml linear gradient composed of pyridine and acetic acid starting at *p*H 3.1, 0.2*M* pyridine; and ending at *p*H 5.0, 2.0*M* pyridine. Thirty-one tryptic peptides have been isolated, of which 14 are arginyl and 17 are lysyl. Most of the peptides contain histidine. It is significant that a "fingerprint" pattern of a tryptic peptide from another crustacean, *Palinurus vulgaris,* also shows about the same number of peptides (*11*). Although the determination of the amino acid composition of each tryptic peptide is not yet complete, this result is consistent with the hypothesis that the minimum functional unit (68,700) consists of two subunits which are very similar and perhaps identical. Since every peptide contains either arginine or lysine, nonspecific cleavage by trypsin does not appear to be a problem.

A molecular weight of 825,000 was previously found for lobster hemocyanin when sedimentation and diffusion coefficients and partial specific volume measurements were used (*13*). We assume that the molecule is composed of 12 subunits, each of molecular weight 68,700. However, the copper content (*14*) of 21 of our samples was 0.175 ± 0.008 percent by weight. The minimum functional unit on this basis would have a molecular weight of 72,600. No significant loss of copper was observed after prolonged dialysis except in calcium-free buffers at *p*H 9.3. Under these conditions hemocyanin slowly lost about one-half of its copper. This observation suggests that the two copper atoms in each binding site may be linked to the protein differently.

If the 4*S* unit contains two polypeptide chains and two copper atoms, one copper atom might be bound by each chain. Each pair of copper atoms is associated with the binding of one oxygen molecule. Perhaps, therefore, each oxygen molecule is bound at a site between two polypeptide chains.

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References and Notes

1. L. T. Hogben, *Brit. J. Exp. Biol.* **3**, 225 (1926); E. Stedman and E. Stedman, *Biochem. J.* **20**, 949 (1926); T. Svedberg and K. O. Pedersen, *The Ultracentrifuge* (Oxford Univ. Press, Oxford, 1940), p. 355; R. Lontie, *Mededel. Vlaam. Chem. Ver.* **16**, 110 (1954); J. R. Redmond, *J. Cell. Comp. Physiol.* **46**, 209 (1955); E. F. J. van Bruggen, E. H. Wiebenga, M. Gruber, *J. Mol. Biol.* **4**, 1 (1962); Ö. Levin, *Arkiv Kemi* **21**, 15 (1963); K. E. Van Holde and L. B. Cohen, *Biochemistry* **3**, 1803 (1964).
2. J. L. Larimer and A. F. Riggs, *Comp. Biochem. Physiol.* **13**, 35 (1964).
3. R. O. Ashby and M. Roberts, *J. Lab. Clin. Med.* **49**, 958 (1957).
4. M. P. Printz, *Fed. Proc.* **22**, 291 (1963).
5. J. Wyman, in *Advanc. Protein Chem.* **19**, 223 (1964).
6. A. Rossi-Fanelli, E. Antonini, A. Caputo, *J. Biol. Chem.* **236**, 397 (1961); E. Antonini, *Physiol. Rev.* **45**, 123 (1965).
7. S. R. Anderson and G. Weber, *Biochemistry* **4**, 1948 (1965).
8. A. F. S. A. Habeeb, H. G. Cassidy, S. J. Singer, *Biochim. Biophys. Acta* **29**, 587 (1958).
9. O. Smithies, *Biochem. J.* **71**, 585 (1959).
10. D. H. Spackman, W. H. Stein, S. Moore, *Anal. Chem.* **30**, 1190 (1958).
11. G. Nardi, A. Ghiretti-Magaldi, G. Caserta, R. Zito, F. Ghiretti, *Boll. Soc. Ital. Biol. Sper.* **24**, 1845 (1962).
12. R. T. Jones, *Cold Spring Harbor Symp. Quant. Biol.* **29**, 297 (1964).
13. M. A. Lauffer and L. G. Swaby, *Biol. Bull.* **108**, 290 (1955).
14. R. E. Peterson and M. E. Bollier, *Anal. Chem.* **27**, 1195 (1955).
15. S. Moore, *J. Biol. Chem.* **238**, 235 (1963).
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Actinomycin D: Inhibition of Respiration and Glycolysis

Abstract. *Actinomycin D inhibited respiration and anaerobic glycolysis of human leukemic leukocytes and lowered the adenosine triphosphate content of the cells. Inhibitory effects on respiration and on RNA synthesis could not be dissociated from one another over a wide range of drug concentrations. Actinomycin D also impaired protein synthesis, probably by decreasing the availability of adenosine triphosphate and by inhibiting messenger RNA.*

Actinomycin D, a chromopeptide produced by actinomycetes, has been shown to selectively inhibit the DNA-directed synthesis of RNA (*1*); it selectively binds to a native DNA template (*2*). Several recent observations, however, have suggested that actinomycin D may also produce subtle cellular changes that are not explained by inhibition of RNA. Revel, Hiatt, and Revel (*3*) reported progressive inhibitory effects on the synthesis of protein in rat liver after RNA synthesis had been blocked, and Korn (*4*) found that the drug impaired phage maturation without detectable effects on RNA. During our studies on the metabolism of human leukemic cells, we observed significant inhibition of glycolysis and respiration in the presence of actinomycin D.

Human leukemic leukocytes were prepared for testing by the following procedure: 50 ml of heparinized blood was allowed to settle at 4°C, the leukocyte–plasma phase was removed, and crystalline glucose (2 mg/ml) and sodium bicarbonate (1 mg/ml) were added. For studies of energy metabolism (inhibition of oxygen consumption and carbon dioxide production) cell suspensions (2.5 ml) were pipetted into manometer flasks and incubated with shaking at 37°C. Actinomycin D was dissolved in Krebs-Ringer bicarbonate medium and tipped into the