100.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Luck, D. N., and Hamilton, T. H. (1975), *Biochim. Biophys. Acta 383,* 23.
Marzluff, W. F., Jr., and Huang, R. C. C. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1082.
Marzluff, W. F., Jr., Murphy, E. C., Jr., and Huang, R. C. C. (1974), *Biochemistry 13,* 3689.
Marzluff, W. F., Jr., White, E. L., Benjamin, R., and Huang, R. C. C. (1975), *Biochemistry 14,* 3715.
Masui, H., and Garren, L. D. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3206.
Mauck, J. C., and Green, H. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2819.
Mauck, J. C., and Green, H. (1974), *Cell 3,* 171.
McReynolds, L., and Penman, S. (1974), *Cell 1,* 139.
Mowshowitz, D. B. (1970), *J. Mol. Biol. 50,* 143.
Muramatsu, M., Onishi, T., Matsui, T., Kawabata, C., and Tokugawa, S. (1974), *Fed. Eur. Biochem. Soc., Meet., 9th, 1974 33,* 325.
Pogo, B. G. T. (1972), *J. Cell Biol. 53,* 635.
Ramachandran, J., and Suyama, A. T. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 113.
Roeder, R. G. (1974), *J. Biol. Chem. 249,* 249.
Roeder, R. G. (1976), in RNA Polymerase, Losick, R., and Chamberlain, M., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory, p 285.
Rubin, H., and Koide, T. (1975), *J. Cell. Physiol. 86,* 47.
Rudland, P. S. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 750.
Schwartz, L. B., and Roeder, R. G. (1974), *J. Biol. Chem. 249,* 5898.
Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinmann, R., and Roeder, R. G. (1974), *J. Biol. Chem. 249,* 5889.
Seifart, K. H., and Benecke, B. J. (1975), *Eur. J. Biochem. 53,* 293.
Seifart, K. H., Benecke, B. J., and Juhasz, P. P. (1972), *Arch. Biochem. Biophys. 151,* 519.
Sklar, V. E. F., and Roeder, R. G. (1976), *J. Biol. Chem. 251,* 1064.
Udvardy, A., and Seifart, K. H. (1976), *Eur. J. Biochem. 62,* 353.
Webster, R. A., and Hamilton, T. H. (1976), *Biochem. Biophys. Res. Commun. 69,* 737.
Weidman, E. R., and Gill, G. N. (1976), *J. Cell. Physiol.* (in press).
Weil, P. A., and Blatti, S. P. (1976), *Biochemistry 15,* 1500.
Weinmann, R., and Roeder, R. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790.
Yu, F.-L. (1974), *Nature (London) 251,* 344.
Yu, F.-L. (1975), *Biochem. Biophys. Res. Commun. 64,* 1107.
Yu, F.-L., and Feigelson, P. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2177.

Hemocyanin from the Australian Freshwater Crayfish *Cherax destructor.* Studies of Two Different Monomers and Their Participation in the Formation of Multiple Hexamers†

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ABSTRACT: The molecular weights of four electrophoretic components of the 17S constituent of the freshwater crayfish *Cherax destructor* have been determined by gel electrophoresis. All four have the same molecular weight, about 470 000, and are therefore hexamers of the 5S constituent of molecular weight 70 000–80 000. The hexamers are stable at pH 7.8 but dissociate at pH 10, upon removal of calcium ions, into the two monomers M_1 and M_2. These monomers are slowly broken down at pH 10, apparently by an enzyme present in serum. The lability thus demonstrated provides a possible explanation for the findings, by some previous investigators, of fragments suggesting that the subunit molecular weight of arthropod hemocyanins was considerably less than 75 000. Evidence for differences in the lability and proteolytic cleavage pattern between M_2 and M_1 is presented. Studies at pH 7.8 in the presence of calcium ions demonstrate that both M_1 and M_2 can form hexamers, the one formed by M_1 having the highest negative charge and that by M_2 the lowest. The proportions of M_1 and M_2 evidently vary in individual crayfish, the variability accounting for the observation that the number of hexamer bands visible on polyacrylamide gels ranges from four to the maximum of seven possible from combinations of two different monomers. The four intense hexamer bands always seen with pooled *Cherax* serum samples are satisfactorily accounted for by the observed ratio of M_1 and M_2 of about 3:1 in such samples. Amino acid compositions are given for serum, M_1, the mixture of hexamers containing M_1 and M_2, and the dimer M_3', a component not involved in the formation of hexamers. Tryptic peptide maps of different hexamers enriched in either M_1 or M_2 are also presented and discussed.

In a previous communication (Murray and Jeffrey, 1974), it was shown that hemocyanin from the crustacean *Cherax destructor* may be regarded as a typical arthropod hemocyanin with respect to the copper content and the molecular weight of the monomer, the types of aggregates it forms, and the dependence of the aggregation upon pH and divalent metal ions. Two different monomeric forms, denoted M_1 and M_2, were seen on polyacrylamide gels at pH 10 and, it appeared, were

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involved in the formation of the 17S constituent of hemolymph. Another species, M_3, of about the same monomer molecular weight, occurring as a dimer at pH 10 in the absence of reducing agent, plays a part in the formation of more highly aggregated constituents. Subunit heterogeneity is evidently not uncommon in arthropod hemocyanins, having been demonstrated, for example, in the crabs *Carcinus maenas* (Busselen, 1970) and *Cancer magister* (Carpenter and Van Holde, 1973), in the ghost shrimp *Callianassa californiensis* (Roxby et al., 1974) and in the horseshoe crab *Limulus polyphemus* (Sullivan et al., 1974). It is of interest therefore to enquire as to the nature of the heterogeneity, its relationship to the aggregation behavior, and ultimately, its significance to the function of hemocyanin as an oxygen carrier. We present here the results of studies of some properties of monomers M_1 and M_2 of *C. destructor* hemocyanin and of their interactions in forming 17S constituent, one of the two major aggregated forms occurring in the hemolymph of this animal.

Experimental Section

Preparation of Hemocyanin Constituents. Serum was prepared from *C. destructor* hemolymph as described previously (Murray and Jeffrey, 1974) and stored under toluene at 5 °C. Usually the serum from several animals was pooled, but some of the experiments referred to later were carried out with the serum obtained from a single crayfish.

The 17S constituent was isolated by gel filtration on Sepharose 6B. One milliliter of serum which had been dialyzed overnight against 0.02 M $CaCl_2$–0.05 M Tris[1] adjusted to pH 7.8 with HCl was applied to a 2.5 cm × 40 cm column and eluted with the same buffer at a flow rate of 10 ml/h. The elution profile showed three peaks, the second of which was concentrated and rechromatographed to give a solution of the 17S material (Murray and Jeffrey, 1974, Figure 2).

The monomer denoted M_1 was prepared by applying 1 ml of serum which had been dialyzed overnight or longer against 0.1 mM EGTA–0.05 M glycine adjusted to pH 10 with NaOH to a 2.5 cm × 40 cm column of Sephadex G-200 equilibrated with the same buffer. Elution at a flow rate of 10 ml/h resulted in a profile with three peaks, the slowest of which was concentrated and rechromatographed to yield electrophoretically pure M_1 (Murray and Jeffrey, 1974, Figure 5). Pure M_1 can also be prepared by column electrophoresis on polyacrylamide gel using the LKB 7900 Uniphor apparatus and the same buffer as in the Sephadex chromatography.

Attempts were made to prepare monomer M_2 using ion-exchange chromatography on both QAE-Sephadex A-50 and DEAE-cellulose, and column electrophoresis on both Sephadex G-200 and polyacrylamide at pH 10 in buffers containing 0.1 mM EGTA. Although M_2 is observed well resolved from M_1 in serum run for 2 h on 7-cm polyacrylamide gels at pH 10, and in peak 3 material from the first chromatography of serum at pH 10 on Sephadex G-200, it has so far not been possible to prepare M_2 as a pure stable component. An explanation for the failure to isolate M_2 is given in the Results and Discussion section. However, hexamers enriched in M_1 or M_2 could be prepared using an LKB 7900 column electrophoresis system. A 10-cm column of 4% polyacrylamide gel in 0.025 M Tris, pH 7.8, containing 0.1 mM EGTA was used. The reservoir was 1.5 l. of the same buffer and crosswise pumping of anode and cathode buffers was employed. After 20 min of preelectrophoresis at 230 V, up to 70 mg of serum protein, dialyzed overnight against the buffer, was applied to the column, the voltage was applied, and the emerging protein was eluted with buffer pumped at 10 ml/h.

Polyacrylamide Gel Electrophoresis. All experiments were carried out at 20 °C in a water-cooled Buchler apparatus using gels 7-cm long and continuous buffer systems. The acrylamide to *N,N'*-methylenebisacrylamide ratio was 40:1 and the gels were polymerized with 0.1% *N,N,N',N'*-tetramethylenediamine and 0.067% ammonium persulfate. At pH 7.8 the buffer was 0.05 M Tris adjusted to pH 7.8 with HCl and gels of 4% acrylamide solution were used. At pH 10.1 the buffer was 0.05 M glycine adjusted with NaOH and containing 0.1 mM EGTA, and 6% gels were used. All gels were given about 15 min of preelectrophoresis at 5 mA/gel before loading protein samples of volume 5–20 μl and approximate concentration 1 mg/ml in 10% glycerol. Electrophoresis was then carried out for 10 min at 2 mA/gel followed by 5 mA/gel for about 1.5 h at pH 10.1 or 2 h at pH 7.8. Gels were stained for half an hour with 0.05% Coomassie brilliant blue in 25% isopropyl alcohol–10% acetic acid and then covered with 7% acetic acid and left to stand overnight before destaining in a Canalco horizontal destainer. Some gels were also stained overnight with periodic acid and Schiff's reagent for the presence of carbohydrate as described by Smith (1960).

Molecular weight determinations were made with the use of the Ferguson (1964) relationship

$$\log R_f = \log (R_f)_0 + K_R T$$

where R_f is the mobility of a protein relative to that of a tracking dye (bromophenol blue), T is the total gel concentration, K_R is the retardation coefficient, and $(R_f)_0$ is the R_f value at a gel concentration of zero. Gels of acrylamide concentration between 4 and 6% were used and, as the mobility of the hemocyanin constituents of interest was much lower than that of the tracking dye, electrophoresis was continued after the emergence of the tracking dye from the end of the gel in order to observe measurable distances of migration. By noting the time taken for the tracking dye to emerge and the time at which the run was ended, it was possible to estimate the distance which the dye would have travelled in the complete run and, hence, the R_f value for a particular protein band. The standard proteins used for calibration were bovine serum albumin monomer, dimer, and trimer, urease, and sulfite-modified urease.

Dialysis, Amino Acid Analysis, and Peptide Maps. Protein solutions were dialyzed in machined Perspex cells of circular cross section in which two compartments each of volume about 1.5 ml were separated by the dialysis membrane and could be loaded and emptied with a syringe. The 2.5-cm diameter membranes were cut from 24/32 Visking dialysis tubing which had been washed with 3% acetic acid at 60 °C followed by copious glass-distilled water. The cells were filled, using sterile precautions as far as possible, and were circulated slowly in a water bath at 20 °C on an apparatus driven by an electric motor, and the outside solution was changed daily for 15 days and monitored for amino acids. The dialysate was made 6 M in HCl by the addition of an equal volume of concentrated acid and hydrolyzed prior to amino acid analysis.

In a separate dialysis experiment, the combined dialysates from 8 days dialysis of M_1 were lyophilized and dissolved in 0.5 ml of 0.5 M HCl to give a solution of pH 9 which was applied to a 1.5 × 55 cm column of Sephadex G-25 equilibrated with 0.05 M ammonium bicarbonate, and washed in with the same buffer. On elution with buffer at 10 ml/h, the elution

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; DEAE, diethylaminoethyl.

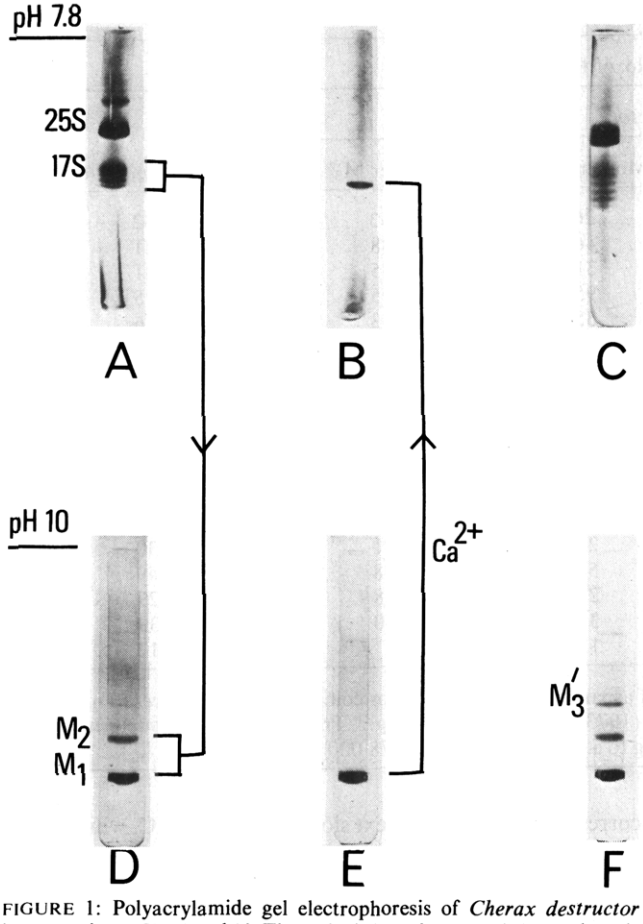

FIGURE 1: Polyacrylamide gel electrophoresis of *Cherax destructor* hemocyanin at pH 7.8 and 10. The cathode is at the top and electrophoresis is from top to bottom. (A) Pooled serum from 20 to 30 animals showing the four main bands of the 17S (hexamer) constituent. (B) The single hexamer band obtained after reconstituting monomer M_1 at pH 7.8 + 0.04 M Ca^{2+}. (C) Serum from a single animal showing six to seven bands for the 17S constituent (electrophoresis continued for longer than in A). (D) The isolated 17S constituent at pH 10 after dissociation at pH 10 in 0.1 mM EGTA showing bands due to the resulting two monomers. (E) Monomer M_1 isolated from the mixture of the two monomers at pH 10. (F) Whole serum at pH 10 showing the presence of dimer M_3' which arises from dissociation of aggregated forms larger than hexamers.

profile was obtained using the absorbance measured at 230 nm. The eluate was pooled to give two fractions and after concentration subjected to peptide mapping on Whatman 3MM paper; the first separation was high-voltage electrophoresis at pH 4.7 (25 ml of pyridine–25 ml of glacial acetic acid made up to 1 l. with water) at 4 V/mm for 1.5 h. This was followed by ascending chromatography (1-butanol–acetic acid–pyridine–water in proportions 15:3:10:12 v/v) for 20 h. The peptides were detected by staining with fluorescamine.

Amino acid analyses were performed with a Beckman 120C analyser by the method of Spackman et al. (1958). Samples were hydrolyzed for 22 h at 110 °C in evacuated sealed tubes containing 2–4 ml of 6 M HCl (Crestfield et al., 1963) and 1% (v/v) aqueous phenol; HCl was removed by rotary evaporation. Methionine and half-cystine were determined as methionine sulfone and cysteic acid, respectively, on samples which had been oxidized with performic acid (Hirs, 1956).

Tryptic peptide maps of hexamers enriched in either M_1 or M_2 were prepared as follows: the proteins and any contaminating proteolytic enzymes were denatured by oxidation with performic acid (Hirs, 1956) and then suspended in 0.5% ammonium bicarbonate pH 8 for digestion by trypsin 2% w/w at 37 °C for 4 h. The small amount of material with limited solubility at pH 4 was removed by centrifugation and the soluble peptides were lyophilized prior to electrophoresis and chromatography as above. Peptides were detected by staining with ninhydrin.

TABLE I: K_R Values and Proportions of *C. destructor* Hemocyanin Hexamers.

Hexamer	Calcd Percentage[a]	Obsd Percentage[b]	K_R[c]
$(M_1)_6$	22	21	0.215
$(M_1)_5(M_2)_1$	38	36	0.217
$(M_1)_4(M_2)_2$	27	32	0.216
$(M_1)_3(M_2)_3$	10	11	0.215
$(M_1)_2(M_2)_4$	2		
$(M_1)_1(M_2)_5$	0.2		
$(M_2)_6$	0.01		

[a] The proportion of each hexamer expected was calculated on the basis of the measured ratio of M_1:M_2, 7:2, found when a sample of pooled serum exhibiting four hexamer bands was dissociated at pH 10, and on the assumption that all of the possible hexamers are formed with equal probability. [b] The proportions of the hexamer electrophoretic bands observed in a sample in which the proportions of monomer M_1 and M_2 were in the ratio 7:2. The percentage of each hexamer band was estimated by photodensitometry on a photographic negative of the gel. [c] The K_R values were measured by gel electrophoresis as described in the text for the four hexamer bands present in sufficient intensity to allow application of the technique. The mean value of the K_R's corresponds to a molecular weight of 470 000.

Results and Discussion

The Electrophoretic Heterogeneity of the 17S Constituent. The electrophoretic pattern at pH 7.8 given by pooled *C. destructor* serum is shown in Figure 1A. When the constituent with a sedimentation coefficient of 17 S is isolated and run on gels under the same conditions, the four bands indicated on the figure are observed. Determination of the K_R values in the Ferguson (1964) relation for each of these bands by linear least-squares fitting of results from electrophoresis experiments on whole serum at different gel concentrations gave the results listed in Table I. It is evident that all of the bands have the same K_R (0.216 ± 0.001) which, from the calibration graph with proteins of known molecular weight, corresponds to a molecular weight of 470 000. The limitations of the calibration procedure suggest that this value could be in error by up to about 10%. Nevertheless the precision in the measurements of the K_R's for the four observed bands is equivalent to a difference in molecular weights of only ±3000 and indicates that the molecular weights of the components giving rise to the bands are identical. When the 17S constituent is dissociated (pH 10 in the presence of 0.1 mM EGTA) the two bands denoted M_1 and M_2 are obtained (Figure 1D) and these have molecular weights of 70 000 and 72 000, respectively, by polyacrylamide gel electrophoresis. The difference is not significant in terms of the experimental error associated with the technique; all that can be said is that the molecular weights of M_1 and M_2 are either identical or very similar and are close to 70 000. An accurate determination of the molecular weight of M_1 by the meniscus depletion sedimentation equilibrium technique, in fact, gave a value of 74 000 for this hemocyanin monomer (Murray and Jeffrey, 1974). Comparison of the molecular weight 470 000 estimated by gel electrophoresis for the four bands of the 17S constituent with that of the monomers which comprise it (taken as 74 700) reveals that the four bands are hexamers. If it is assumed that both M_1 and M_2 are

TABLE II: Amino Acid Composition of Hemocyanin Samples.

	No. of Residues/mol				
	Cherax destructor[a]				
Amino Acid	Serum	17S ($M_1 + M_2$)	Monomer M_1	Dimer M_3'	*Cancer magister*[b]
Lys	31	28	26	33	32
His	45	47	46	38	41
Arg	33	32	31	35	31
Asp	95	95	95	95	95
Thr	34	35	35	33	36
Ser	38	39	39	35	36
Glu	67	66	67	66	68
Pro	32	28	28	32	32
Gly	42	42	41	36	40
Ala	39	40	39	40	38
Cyst(e)ine	2	Tr	0	2-3	3
Val	49	52	53	40	48
Met	5	4	4	5	16
Ile	29	27	26	31	30
Leu	54	52	52	58	49
Tyr	26	28	27	28	29
Phe	39	36	37	40	39
Trp[c]	12	12	12		12

[a] Values are the mean of two determinations calculated on the basis of aspartic acid equal to 95 for comparison with *Cancer magister*. Tr = Trace. [b] Values for *Cancer magister* from Carpenter and Van Holde (1973) in residues/78 000 g. Tryptophan was not determined for *C. destructor;* the value was set equal to that for *Cancer magister* leading to a molecular weight of 78 000 for M_1.

equally competent to form hexamers there are seven combinations possible, $(M_1)_6$, $(M_1)_5\cdot(M_2)_1$, $(M_1)_4\cdot(M_2)_2$, $(M_1)_3\cdot(M_2)_3$, $(M_1)_2\cdot(M_2)_4$, $(M_1)_1\cdot(M_2)_5$, and $(M_2)_6$. Knowledge of the proportions of M_1 and M_2 in the 17S constituent then allows calculation of the proportions of each of these hexamers. The results of such a calculation for the proportions of M_1 and M_2 estimated to be present in the 17S constituent from pooled serum are given in Table I. Four hexamers, those containing the four highest proportions of M_1, account for 97% of the total hexamer and moreover their relative amounts correspond with those estimated experimentally. This result suggests that the fastest band in Figure 1A represents $(M_1)_6$ followed by $(M_1)_5\cdot(M_2)_1$, $(M_1)_4\cdot(M_2)_2$, and $(M_1)_3\cdot(M_2)_3$. Some serum from individual crayfish does show more than four distinguishable bands with mobilities corresponding apparently to hexamers, for example, as indicated in Figure 1C, gels showing up to the expected seven have been observed. This might mean that individual animals have different proportions of M_1 and M_2 in their hemolymph and/or that some of the M_2 originally present can be lost during manipulation of samples.

The difficulty in confirming the scheme proposed to account for the origin of the electrophoretic heterogeneity of the 17S constituent resides in our inability to isolate the monomer M_2. However, two further pieces of evidence may be cited in support of the proposal. First, if purified monomer M_1 is dialyzed at pH 7.8 in the presence of Ca^{2+} and the resulting solution run on polyacrylamide, the pattern shown in Figure 1B is obtained. Comparison with Figure 1A reveals that, under these conditions, M_1 forms an aggregate with the mobility of the fastest hexamer band, confirming that the band in question is indeed $(M_1)_6$. Secondly, when a section of polyacrylamide gel corresponding to the position of M_2 in Figure 1D was cut out, macerated, extracted, and concentrated, and the resulting solution dialyzed at pH 7.8 in the presence of Ca^{2+} and run on polyacrylamide, a band running in the same position as the slowest hexamer band on Figure 1C was obtained. This seems to be, as expected, $(M_2)_6$. There was also a suggestion of a band corresponding with the next slowest in Figure 1C, which could result from combination of M_2 with a trace of M_1 retained from the gel extraction to give $(M_1)_1\cdot(M_2)_5$.

Amino Acid Analysis and Dialysis at pH 10 and pH 7.8. The results of amino acid analyses carried out on hydrolysates of *C. destructor* serum, the 17S constituent (containing M_1 and M_2), and monomer M_1, are reported in Table II. It could be noted, first, that comparison of the compositions of *Cherax destructor* serum and *Cancer magister* serum (Carpenter and Van Holde, 1973) reveals that the hemocyanins from these two crustacean species are very similar. Secondly, no significant differences could be observed between the compositions of the 17S constituent and monomer M_1, indicating that M_2 must be quite similar to M_1. Any small differences which do exist would of course be very difficult to pick up in a comparison of this kind since M_2 represents only about one-fourth of the 17S constituent. The difficulty was reduced somewhat by comparing analyses performed on the 17S constituent enriched in M_1 and M_2, respectively (cf. Figure 3). Here the amounts of lysine, valine, and isoleucine differed by 10% or more between the two fractions. There do seem to be significant differences between the compositions of *C. destructor* serum and that of either the 17S constituent or M_1. These differences are consistent with the presence in serum of another component which has previously been designated dimer M_3' (Murray and Jeffrey, 1974) and whose composition is given in Table II. It can be seen that dimer is higher than serum in those residues for which serum is higher than the 17S constituent and lower than serum in those for which serum is lower than the 17S constituent. The properties of this component are under investigation and will be the subject of a future communication.

The failure to isolate monomer M_2 from the mixture of the two monomers produced by dissociating the hexamers at pH 10 in the presence of 0.1 mM EGTA suggested that M_2 is unstable under such conditions. In order to test this idea and to explore further the relationship between M_1 and M_2, hemocyanin solutions were dialyzed against two volumes of

TABLE III: Amino Acid Composition of External Solution after 2 Days of Dialysis of the M_1 and 17S Constituents of Hemocyanin at pH 10.

	No. of Residues[a]			
Amino Acid	Monomer M_1	Δ^b	17S Constituent	Δ^b
Lys	32	+6		
His	43	−3		
Arg	33	+2	53	+21
Asp	95	0	95	0
Thr	35	0	33	−2
Ser	41	+2	33	−6
Glu	66	−1	57	−8
Pro	30	+2	39	+11
Gly			41	−1
Ala			41	+1
Cyst(e)ine	0	0	0	0
Val	47	−6	45	−7
Met	4	0	4	0
Ile	29	+3	41	+14
Leu	48	−4	65	+13
Tyr	28	+1	35	+7
Phe	39	+2	49	+13

[a] The number of residues is calculated on the basis that aspartic acid is equal to 95. [b] The symbol Δ refers to the difference [number of residues in the external solution minus number in the original solution (Table Ii)]. Figures are not given for lysine and histidine for the 17S constituent, because it was found subsequently that the Tris buffer in which this constituent was dissolved interfered with the determination of these amino acids. Similarly, glycine and alanine figures are not given for M_1, because it was dialyzed in a glycine-containing buffer.

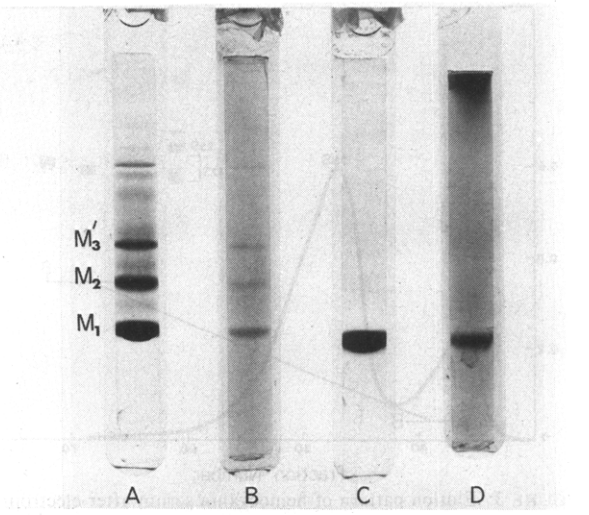

FIGURE 2: Polyacrylamide gel electrophoresis of *Cherax destructor* hemocyanin at pH 10 showing the presence of carbohydrate. (A) Serum stained with Coomassie blue showing the two monomers M_1 and M_2 and the dimer M_3' resulting from dissociation of the aggregated forms. (B) As A but stained for carbohydrate with periodic acid and Schiff's reagent. (C) Monomer M_1 stained with Coomassie blue. (D) Monomer M_1 stained for carbohydrate.

buffer (0.05 M sodium carbonate–sodium bicarbonate, 0.1 mM EGTA, pH 10) for various lengths of time and the outside solution was examined by amino acid analysis after hydrolysis in 6 M HCl. Experiments of this kind with bovine serum albumin and β-lactoglobulin showed no detectable amino acids outside the membrane after 10 days of dialysis. However, in separate experiments with the hemocyanin hexamers and with monomer M_1, all of the amino acids occurring in these components were found in the outside buffer after only 2 days and continued to appear over the 15-day period for which the dialysis was continued.

The composition of the outside solution collected over the first two days of dialysis is given for monomer M_1 and the 17S constituent in Table III. Two points should be made before drawing any conclusions from the results quoted in the table. First, it must be remembered that the amino acids were determined on hydrolysates of solutes which had dialyzed through a membrane and the compositions will therefore not necessarily correspond with those of the whole proteins which are being dialyzed. It is worth noting also that the sizes of the fragments escaping through the membrane could range from single amino acids up to peptides of molecular weight several thousand. Secondly, the figures in Table III have been arbitrarily calculated for purposes of comparison on the basis that the aspartic acid content corresponded to 95 residues as was done for the intact proteins (Table II). Inspection of Table III reveals that the amino acid composition of the external solution after dialysis of M_1 is, in fact, so similar to that of M_1 itself that it is reasonable to assume that M_1 is broken completely into dialyzable fragments. However, in the case of the 17S constituent, comparison of the composition of the external solution after 2 days of dialysis with that of the solution originally inside the membrane shows considerable differences in some of the amino acids. Since the 17S constituent contains M_2 as well as M_1, the difference in the behavior on dialysis at pH 10 must arise from the presence of M_2. In addition, because no such difference could be detected from a comparison of the amino acid compositions of intact 17S constituent and monomer M_1 (Table II), the dialysis experiments indicate that M_2 is broken down in the solution of hexamers more readily than M_1—the external solution at this early stage of dialysis containing a higher proportion of amino acids derived from M_2 than were present in the original 17S constituent. In accordance with this reasoning polyacrylamide gel electrophoresis of the 17S constituent showed, after 2 days of dialysis, a pronounced decrease in the proportion of M_2 with respect to M_1 and on further dialysis it was observed that M_2 disappeared completely.

To characterize further the breakdown products at pH 10, the dialysate from 8 days of dialysis of M_1 was applied to a column of Sephadex G-25. The elution profile indicated the presence of a range of peptides of varying molecular weights. Two-dimensional separation of these peptides on paper showed only very faint fluorescence with fluorescamine indicating breakdown to a very large number of different peptides. Combined dialysates from 8 days of dialysis of the 17S constituent were also lyophilized and applied to the G-25 column. The eluate from this experiment again indicated the presence of peptides over a wide molecular weight range but was noticeably different from that of M_1 (a greater proportion of peptides of intermediate size). The peptide maps from the 17S constituent dialysate showed many extra peptides which stained quite well in comparison with those from the M_1 dialysate. This could be taken to mean that the M_2 component is subject to more uniform (and facile) cleavage by a postulated contaminating proteinase at a large but specific number of bonds. The overall result of the dialysis experiments stresses the apparent differences between M_1 and M_2 and that there does not appear to be a simple relationship between their structures.

Figure 2 shows the results of polyacrylamide gel electrophoresis experiments in which the gels were stained for car-

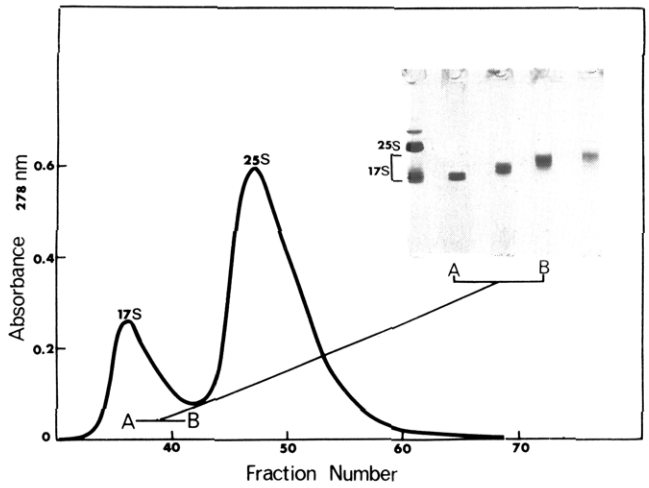

FIGURE 3: Elution pattern of hemocyanin serum after electrophoresis on polyacrylamide gel at pH 7.8 in LKB 7900 apparatus. The inset shows the enrichment in M_2-containing hexamers from A to B. The gel pattern on the left is that of serum at pH 7.8, while that on the right is a fraction from the ascending side of the 25S peak immediately after B in the elution profile showing the 25S material beginning to appear in the effluent.

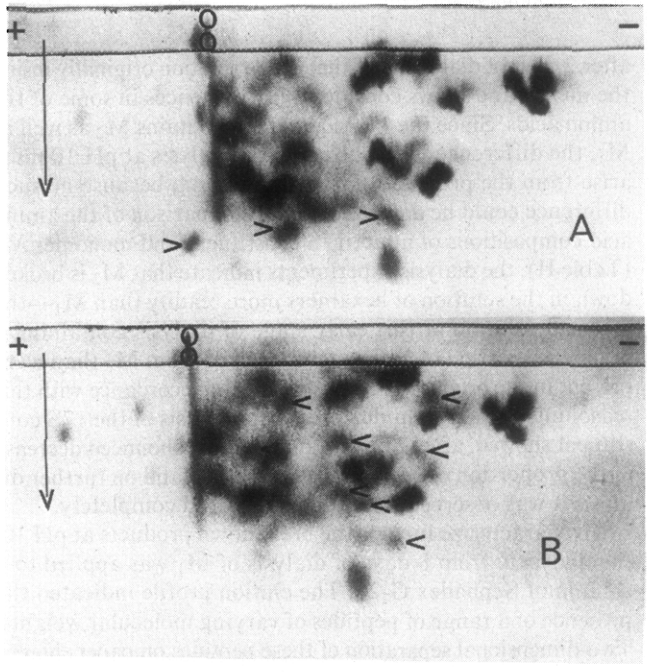

FIGURE 4: Peptide maps of 17S constituent of *Cherax destructor* hemocyanin. The origin and the direction of electrophoresis and chromatography is indicated; for further details, see text. (A) Constituent (17S) enriched in M_1-containing hexamers; (B) constituent (17S) enriched in M_2-containing hexamers. *The arrowed spots are those which appear in* one peptide map but not in the other.

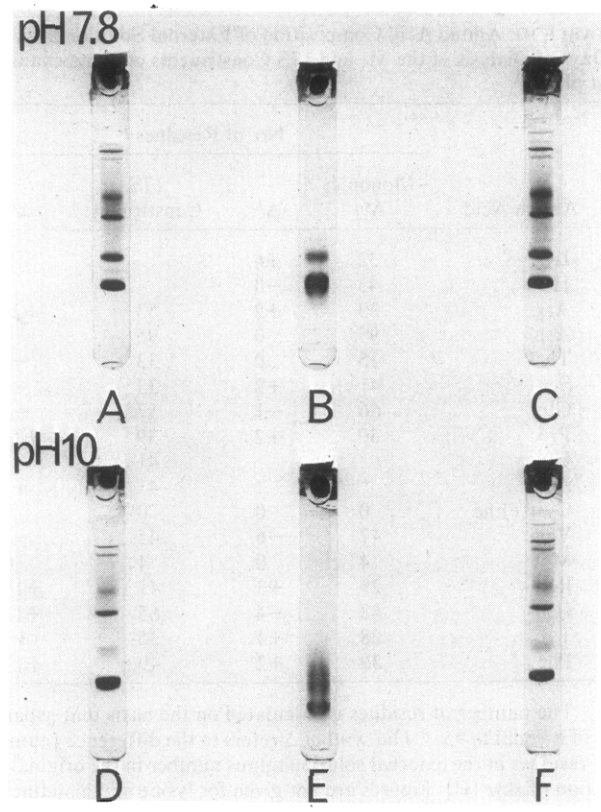

FIGURE 5: Polyacrylamide gel patterns of hemocyanin and β-lactoglobulin A incubated at pH 7.8 and 10 showing proteolytic breakdown of β-lactoglobulin in the presence of hemocyanin. (A and D) Hemocyanin alone; (B and E) β-lactoglobulin alone; (C and F) hemocyanin + β-lactoglobulin. Solutions of the two proteins alone and together were incubated *overnight at 30 °C under nitrogen gas in the presence of 0.1 mM EGTA*–1 mM dithiothreitol at pH 7.8 (0.05 M Tris) and 10 (0.05 M sodium carbonate–sodium bicarbonate), respectively. The concentration of hemocyanin in the incubation mixture was 1.33 mg/ml and of β-lactoglobulin 0.67 mg/ml and, following incubation, 20-μl samples were applied to 8% gels at pH 10 containing 0.1 mM dithiothreitol and 0.1 mM EGTA.

bohydrate. These show that monomer M_1, monomer M_2, and dimer M_3' all contain carbohydrate and evidently there is no substantial difference in the carbohydrate content of M_1 and M_2 since the bands stained for carbohydrate have about the same relative intensities as those stained with Coomassie blue. These experiments, therefore, also imply differences in amino acid composition between M_1 and M_2.

Finally, peptide maps of the 17S constituent enriched in M_1 and M_2, respectively, as described in the Experimental Section, are reproduced in Figure 4. The comparison is made with an M_1-enriched 17S fraction rather than with pure M_1 because the latter can only be prepared at pH 10 where some breakdown may occur before the digestion with trypsin. The elution pattern from the column electrophoresis and polyacrylamide gels of the fractions showing progressive enrichment in M_2 are shown in Figure 3. The peptide maps show an overall similarity, with each having a few ninhydrin-positive spots not found in the other. Amino acid analyses of an arbitrary selection of the strongly staining peptides confirmed their similarity in each map. The extra peptides were found to contain less material than those which were common to the two maps but the composition of each included lysine or arginine consistent with them being true tryptic peptides and not a result of cleavage by the suspected contaminating proteinase. The differences between M_1 and M_2 suggested by the dialysis experiments are less apparent from the tryptic peptide maps; nevertheless there are differences. Unfortunately it is not possible to say anything unequivocally about the relationship between the monomers because of the obvious difficulties of interpretation of results obtained with mixtures. It does not seem profitable to pursue the enquiry further with such mixtures and accordingly renewed efforts are being made to isolate M_2 to allow direct comparison of its composition and structure with that of M_1.

Dialysis experiments demonstrating the breakdown of the two monomers at pH 10 have been described and the presence of a proteolytic enzyme in serum has been assumed to be the

likely cause. Direct evidence for the validity of the assumption is presented in Figure 5 which shows that, when β-lactoglobulin A is incubated with *Cherax destructor* serum at pH 10 or 7.8, the protein is broken into fragments too small to be detected on polyacrylamide gels run under standard conditions. In these experiments it was necessary to perform the incubation under nitrogen gas and in the presence of 1 mM dithiothreitol to prevent aggregation of fragments through disulfide interchange. When the 17S constituent was dialyzed in a cell at pH 7.8 (0.05 M Tris adjusted with HCl), in contrast to the results of pH 10, no amino acids were found in the outside solution even after 12 days of dialysis. Preincubation of hemocyanin with the enzyme inhibitors parahydroxymercuribenzoate (0.15 mM) and phenylmethylsulfonyl fluoride (0.3 mM) at pH 7.8 and their inclusion in the experiments at pH 10 failed to produce any significant change in the rate at which hemocyanin was broken down. Likewise iodoacetamide (0.26 mM) did not prevent the disappearance of M_2, as monitored by polyacrylamide gel electrophoresis, in samples of hemocyanin at pH 10. Thus as yet it has not been possible to prevent the proteolysis which we have demonstrated to occur.

Conclusions

The results presented here show that both monomers M_1 and M_2 from *C. destructor* serum are labile at pH 10, M_2 being broken down more readily than M_1. The greater instability of M_2 together with its presence in lower amount than M_1 accounts for the failure to purify it, particularly since the standard method for obtaining M_1 and M_2 has been to dissociate the hexamers at pH 10. Both monomers are capable of forming hexamers when Ca^{2+} is present, the number of hexamer bands which are seen on polyacrylamide gels being dependent on the proportions of M_1 and M_2 present. This ratio seems to vary in individual crayfish but its limits have not been established because of the problems associated with the lability of M_2. However, there always seems to be less M_2 than M_1 and, judging by the results with serum pooled from up to 30 animals, an average value for the ratio is about three parts of M_1 to one of M_2. It is estimated, from the experimental error in determining the molecular weights of the four hexamers investigated, that their molecular weights could differ by up to 6000 before a measurable difference was reliably detected. This means that the molecular weights of M_2 and M_1 could differ by up to about 2000 without being reflected as an observable difference in the molecular weights of $(M_1)_6$ and $(M_1)_3 \cdot (M_2)_3$.

The finding that *Cherax destructor* hemocyanin contains two different monomers with molecular weights differing by perhaps 2000 in the molecular weight range 70 000–80 000 finds a parallel in the results of Carpenter and Van Holde (1973) with *Cancer magister* hemocyanin. Some differences in properties which could be noted, however, are first that, whereas the proportions of the two monomers found in *Cancer magister* appear to be about equal, in *Cherax destructor* they occur in a ratio of about 3:1. Secondly, isomeric forms of the monomers and hexamers of *Cherax destructor* hemocyanin are resolved on polyacrylamide gels but those from *Cancer magister* are not. The demonstrated proteolytic activity present in *Cherax destructor* hemocyanin at pH 10 reinforces the conclusion of Carpenter and Van Holde (1973) that results obtained by previous workers which seemed to show the existence of subunits smaller than that of molecular weight 75 000 in arthropod hemocyanins could be explained by the presence of fragments produced by hydrolysis.

The functional significance of the presence of monomer variants in hemocyanins may well be, as suggested by the work of Sullivan et al. (1974) with *Limulus polyphemus* hemocyanin, to provide the organism with flexibility of respiratory response to varying environmental conditions. We hope to investigate this question more fully by oxygen-binding studies on whole *C. destructor* serum and on individual constituents which can be isolated from it. The further elucidation of interactions between constituents is necessary in this connection, in particular the relationship of the so-called "dimer" (M_3' in Murray and Jeffrey, 1974) to M_1 and M_2, since its presence seems to be required for the formation of higher aggregates than hexamers.

References

Busselen, P. (1970), *Arch. Biochem. Biophys. 137,* 415.
Carpenter, D. E., and Van Holde, K. E. (1973), *Biochemistry 12,* 2231.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238,* 622.
Ferguson, K. A. (1964), *Metabolism 13,* 985.
Hirs, C. W. H. (1956), *J. Biol. Chem. 219,* 611.
Murray, A. C., and Jeffrey, P. D. (1974), *Biochemistry 13,* 3667.
Roxby, R., Miller, K., Blair, D. P., and Van Holde, K. E. (1974), *Biochemistry 13,* 1662.
Smith, I. (1960), Chromatographic and Electrophoretic Techniques II, 200, Heinemann.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Sullivan, B., Bonaventura, J., and Bonaventura, C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2558.