*Results.* There were no statistically significant differences between the 3 treatment conditions relative either to the Hypnotic Induction Profile or to the Stanford Sleepiness Scale (table). There were no reports of unusual subjective states or sensations, and the subjects were unable to identify when they received naloxone.

*Discussion.* The data disclaim our hypothesis that endorphins might be released during hypnosis in such a way that they could influence the depth of hypnotic trance. The failure of the large dose of naloxone to change the scores of the Stanford Sleepiness Scale represents new data confirming in a quantitative way the lack of effect of this dose of naloxone in relation to levels of alertness. Our observation that subjects were unable to identify when they received naloxone corroborates further the lack of subjective effects of this drug in individuals who are not using narcotics. Given antagonism of the endorphin receptor, the pharmacological blandness of naloxone remains a major stumbling block to hypotheses which seek to relate psychological states to the endorphin system.


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2 Lehmann, H., Nair, N.P., and Kline, N.S., Am. J. Psychiat. *136* (1979) 762.
3 Davis, G.C., and Bunney, W.E., in: Neural peptides and neuronal communication, p.455. Eds E. Costa and M. Trabucchi. Raven Press, New York 1980.
4 Liao, S.J., Yale J. biol. Med. *51* (1978) 55.
5 Wagemaker, H., and Goldstein, L., Sports Med. Phys. Fitness *20* (1980) 227.
6 Colt, E.W., Wardlaw, S.L., and Frantz, A.G., Life Sci. *28* (1981) 1637.
7 Bortz, W.M., Angwin, P., Mefford, I.N., Boarder, M.R., Noyce, N., and Barchas, J.D., New Engl. J. Med. *305* (1981) 466.
8 Levine, J.D., Gordon, N.C., and Fields, H.L., Lancet *2* (1978) 654.
9 Frid, M., and Singer, G., Psychopharmacology *63* (1979) 211.
10 Nasrallah, S.M., Holley, T., and Janowsky, D.S., Lancet *1* (1979) 1355.
11 Spiegel, H., Ann. N.Y. Acad. Sci. *296* (1977) 129.
12 Spiegel, H., and Fleiss, J., Int. J. clin. exp. Hypnosis *24* (1976) 300.
13 Zaks, A., Jones, T., Fink, M., and Freedman, A., J. Am. med. Ass. *215* (1971) 2108.
14 Hoddes, E., Zarcone, V., Smythe, H., Phillips, R., and Dement, W., Psychophysiology *10* (1973) 431.


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## Chromatographic separation of coelomic fluid from *Holothuria polii* (Echinodermata) and partial characterization of the fractions reacting with erythrocytes

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*Summary.* Coelomic fluid preparations from *Holothuria polii* were passed through a Bio-gel A5m column. The 3 separated protein peaks possess hemagglutinating or hemolytic activity against rabbit erythrocytes. Electrophoretic and immunochemical methods showed that 2 identical protein subunits characterized hemagglutinins of different size. Hemolysin differs from hemagglutinin in molecular weight and organization of subunits.

Coelomic fluid from certain echinoderm species possesses naturally occurring hemolytic and also hemagglutinating activity against erythrocytes of several vertebrate species. Chemico-physical investigations of sea-urchin[2], star-fish[3] and holothurian[4] hemagglutinins showed that, except for those of the sea-urchin *Hemicentrotus pulcherrimus*, they are protein or protein-like substances. The same methods also suggest that hemolysins are thermolabile proteins active, in alkaline medium supplemented with $Ca^{2+}$, in lysing vertebrate erythrocytes[5,6]. In previous papers[4,6] we showed that hemagglutinins and hemolysins from *Holothuria polii* are proteins which differ in some of their chemico-physical properties. The present report concerns a partial molecular characterization of the active fractions obtained by chromatographic separation of the coelomic fluid.

*Materials and methods. Holothuria polii* Delle Chiaje specimens were collected in the Gulf of Palermo. Coelomic fluid was centrifuged at 400×g to remove the cells and then, after dialysis with phosphate buffered saline at pH 7.4 (PBS), stored at −20 °C.

Hemagglutination and hemolysis assays have been previously described[4,6]. The end titer was taken to be the reciprocal of the highest dilution of the coelomic fluid giving a clear agglutination after gentle shaking, or revealing hemolysis (at least 10%).

To determine protein content the Folin-Ciocalteau method as described by Lowry et al.[7] was used. Bovine serum

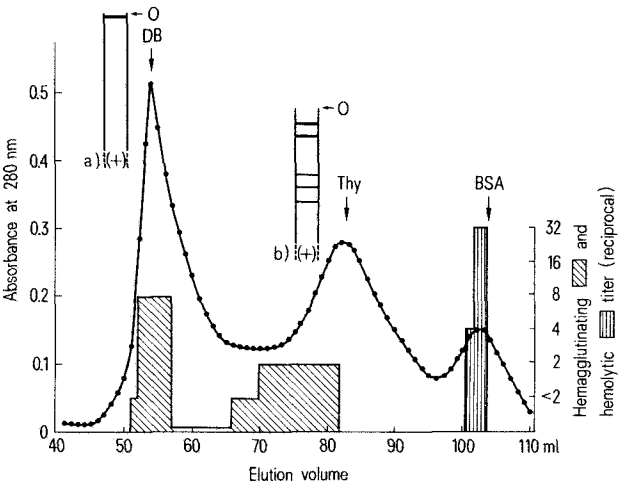


Figure 1. Bio-gel A5m elution pattern of 10-fold concentrated *Holothuria polii* coelomic fluid showing the distribution of hemagglutinating and hemolytic activity. The eluting buffer was phosphate buffered saline pH 7.4, the column size 1.5×90 cm. The elution volumes of dextran blue (DB), thyroglobulin (Thy) and bovine serum albumin (BSA) are indicated. Inset: diagrams which represent developed portions of 7.5% polyacrylamide gel electrophoresis; (a) 1st peak, (b) 2nd peak, (O) origin.

albumin was the reference standard. Each value was expressed as the average of 3 determinations ± SE.

Coelomic fluid was fractionated by gel filtration utilizing Bio-gel A5m (Bio-Rad) columns (1.5 × 90 cm) equilibrated with PBS. Samples were concentrated by ultrafiltration in a Diaflo equipped with a UM 2 membrane (Amicon Corp., Lexington, Mass.) and then dialyzed overnight against the starting buffer. The fractions (1 ml) were monitored for UV absorbancy at 280 nm and tested for hemagglutinating activity. Dextran blue 2000 (Pharmacia), thyroglobulin and bovine serum albumin (Sigma) were used to calibrate the column[8] for molecular weight determination. Disc electrophoresis in 7.5% polyacrylamide gels (PAGE) was performed as described by Davis[9]. The sample (100 µl) was mixed (v/v) in 40% sucrose 5 mM glycine pH 8.3 and deposited on the spacer gel. The electrophoretic run was carried out at 1 mA/tube for 30 min and then at 3 mA/tube for 60 min. Proteins were stained for 1 h with Coomassie blue. To assay the hemagglutinating activity of the protein fractions after electrophoresis, unstained gels were cut by gel slicer; each slice was homogenized and placed overnight in 0.5 ml PBS. The supernatants obtained by centrifuging the homogenates were assayed with rabbit erythrocytes (RE). SDS-PAGE[10,11] was carried out as described by Gavery et al.[12]. Gels containing 5.6% acrylamide and 0.2% N,N-methylenbisacrylamide were used. The samples were dialyzed against 0.02 M Tris-acetate buffer containing 2% β-mercaptoethanol, 2% sodium dodecyl sulfate (SDS) and then heated for 10 min at 100 °C. Electrophoresis was carried out for 4 h at 4 mA/tube. The molecular weight values were obtained in 3 independent experiments. A standard curve was constructed by plotting the electrophoretic mobilities obtained for reference proteins against molecular weights. The reference proteins (Bio-Rad) were: myosin (mol. wt 200,000), β-galactosidase (mol. wt 116,250), phosphorylase B (mol. wt 92,500), bovine serum albumin (mol. wt 66,200), ovalbumin (mol. wt 45,000), carbonic anhydrase (mol. wt 31,000), soybean trypsin inhibitor (mol. wt 21,500), lysozyme (mol. wt 14,400). Antiserum to coelomic fluid of *H. polii* was produced by injecting rabbits with 5 ml coelomic fluid emulsified with an equal volume of Difco Freund's complete adjuvant. Two boosters of coelomic fluid without adjuvant were given on day 30. The animals were bled 10 days afterwards.

Bidimensional immunodiffusion (ID)[13] on 1.5% agarose (Bio-Rad) was performed in mono-bipotassic phosphate buffer 0.03 M + NaCl 0.1 M pH 8. Microimmunoelectrophoresis (IEP)[13,14] was carried out using 1.5% agarose and Tris-barbital sodium barbital buffer pH 8.8. A current of 4 mA/strip was applied for 50 min. Diffusion of the antiserum in ID and IEP was allowed to occur in a moist chamber at room temperature for generally 24–72 h. Dried agarose films were stained with Coomassie blue.

*Results and discussion.* Fresh preparations of 10-fold concentrated coelomic fluid (protein content 1.5 ± 0.5 mg/ml) were passed through a Bio-gel A5m column. As shown in figure 1 the $A_{280}$ profile consisted of 3 major components: the first and second were eluted in the same volumes as dextran blue (mol. wt $5 \times 10^6$) and thyroglobulin (mol. wt 750,000) respectively, the third was eluted just before bovine serum albumin (mol. wt 66,200). Each eluted fraction was tested for biological activity using RE. Most of the hemagglutinating activity was in the single major protein fraction which eluted with the dextran blue. A smaller activity peak came off a few tubes behind the dextran blue and included several fractions preceding the thyroglobulin peak.

Hemolysins against RE were only found in the minor component which was eluted earlier than BSA: the fractions which showed the greatest UV absorbances lysed the erythrocytes even when diluted to 1:32.

Disc electrophoresis experiments showed that the proteins excluded from the Bio-gel column did not enter the 7.5% polyacrylamide gel. This electrophoresis fraction (fig. 1), eluted from the gel slices, contained hemagglutinins. Five protein bands were found after electrophoresis of the 2nd

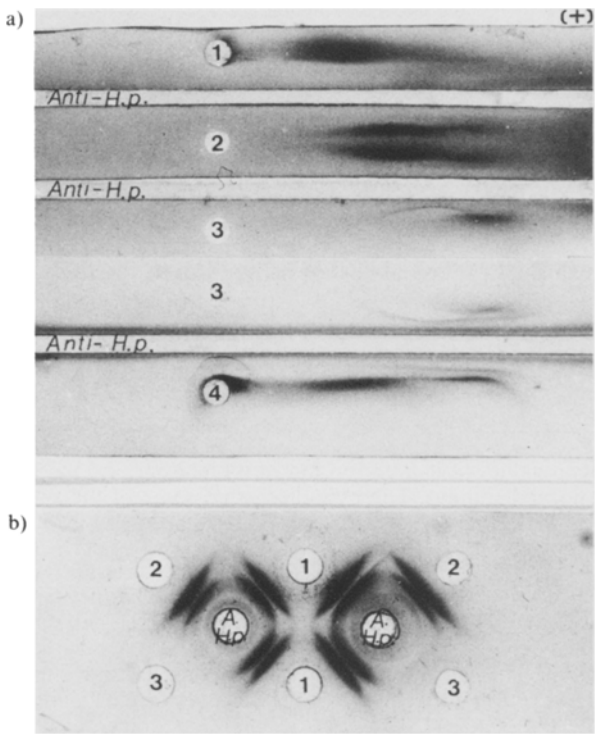


Figure 2. Immunoelectrophoresis (a) and immunodiffusion (b) of the fractions eluted by Bio-gel chromatography: 1st peak (1), 2nd peak (2), 3rd peak (3) and whole coelomic fluid (4) assayed against anti-*Holothuria polii* coelomic fluid antiserum *(Anti-H.p.; A.H.p.)*.

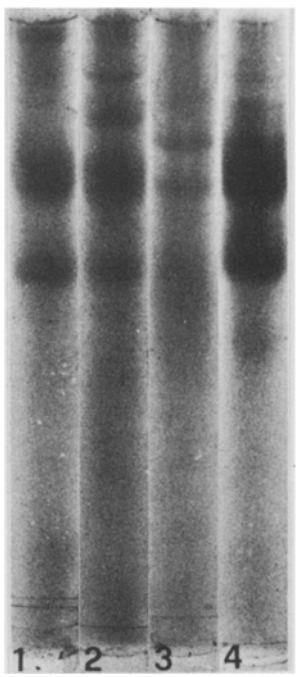


Figure 3. SDS-polyacrylamide gel electrophoresis of the Bio-gel eluted fractions; 1st peak (1), 2nd peak (2), 3rd peak (3), whole *Holothuria polii* coelomic fluid (4). The reference proteins used to calibrate the molecular weight curves are given in the methods.

peak; however, the assay used was not able to show which of them possessed the hemagglutinating activity. Both electrophoretic patterns together were equivalent to the whole coelomic fluid electropherogram. Colored protein bands were not visible in the gel after electrophoresis of the 3rd peak, probably owing to the very low protein concentration.

The pooled fractions of each peak were also investigated with an immunoserum against the whole coelomic fluid. IEP (fig. 2,a) showed that several antigens of differing electrophoretic mobility were contained in the coelomic fluid and characterized each chromatographic component. The fractions eluted with dextran blue, mainly consisting of electrophoretically slow proteins of intermediate mobility, were found in the 2nd peak; 3 distinct anodal immunoprecipitates were typical of the 3rd peak. ID (fig. 2,b) of these reactants confirmed that several immunologically distinguishable components were contained both in the fractions eluted with the dextran blue (at least 3 immunoprecipitates) and in those eluted with thyroglobulin (at least 2 immunoprecipitates). Three very thin arcs, difficult to see, represent the antigenic composition of the low molecular weight fraction. By SDS-PAGE, 2 protein chains, 68,000 and 30–35,000 daltons respectively, were found in the fractions of both the 1st and 2nd peak of the $A_{280}$ profile. The latter also showed molecules of 185–200,000 and 115–130,000 daltons but we do not know whether they are large molecular chains or derivatives produced as a result of treatment during the SDS-PAGE procedure. The 3rd peak showed 2 protein chains of about 90,000 and 68,000 daltons.

The relationship between protein subunits and hemagglutinins was investigated by absorption experiments. Coelomic fluid preparations were absorbed with an equal volume of packed RE to eliminate the anti-RE hemagglutinins. After centrifugation, the supernatant was studied in SDS-PAGE. The electropherogram, compared with that of a non-absorbed sample, showed an evident reduction of the 68,000 and 30–35,000 dalton bands.

The coelomic fluid, frozen at −20 °C for 4–5 weeks, and then passed through a Bio-gel column, apparently lost most of the light molecular weight molecules and no hemolytic activity was found in the fractions of the 3rd chromatographic component. The largest hemagglutinins were sensitive to prolonged freezing (5–6 months): hemagglutinating activity was only found in the component eluted with tyroglobulin.

The result so far obtained show that 2 protein subunits in the *H. polii* coelomic fluid could form hemagglutinin molecules heterogeneous in size, the largest being sensitive to freezing. As suggested in a previous paper[6], the hemolysins differ from the hemagglutinins in that they are smaller, more sensitive to temperature variations and can be distinguished by immunoelectrophoresis. The hemolysins could also contain a 68,000 dalton protein chain; however, further work is needed to clarify whether or not affinity exists between hemolysin and hemagglutinin subunits.

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2 Ryoyama, K., Biol. Bull. *146* (1974) 404.
3 Finstad, C.L., Litman, G.W., Finstad, J., and Good, R.A., J. Immun. *108* (1972) 1704.
4 Parrinello, N., Canicatti, C., and Rindone, D., Boll. Zool. *43* (1976) 259.
5 Ryoyama, K., Biochim. biophys. Acta *320* (1973) 157.
6 Parrinello, N., Rindone, D., and Canicatti, C., Devl comp. Immun. *3* (1979) 45.
7 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
8 Andrews, P., Biochem. J. *96* (1965) 595.
9 Davis, B.J., Ann. N.Y. Acad. Sci. *121* (1964) 404.
10 Shapiro, A.L., Vinuela, A.L., and Maizel, J.Y., Biochem. biophys. Res. Commun. *28* (1967) 815.
11 Weber, K., and Osborne, M., J. biol. Chem. *244* (1969) 4406.
12 Gavery, S.S., Cremer, N.E., and Sussdorf, D.H., Methods in immunology. W.A. Benjamin, Inc., London 1967.
13 Ouchterlony, O., and Nilsson, L.A., in: Handbook of experimental immunology, chapter 19, p.39. Ed. D.W. Weir. Blackwell, Oxford 1973.
14 Scheidegger, J.J., Int. Archs Allergy *7* (1955) 103.

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## Beta-endorphin immunoreactivity in the plasma of patients with the Prader-Labhart-Willi syndrome and their normal siblings

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*Summary.* No significant difference was found in the range or mean values of ir-β-endorphin in the plasma of 6 patients with the Prader-Labhart-Willi syndrome compared to 7 of their normal siblings. The hypothesis that some of the symptoms of the P-L-W syndrome are due to excessive opioid activity is not supported by measurement of peripheral levels of ir-β-endorphin.

Excessive levels of immunoreactive beta-endorphin (ir-β-ep) occur in the pituitary of genetically obese rodents[2] such as *ob/ob* and *fa/fa* as well as in the plasma of obese hirsute women[3]. These forms of obesity involve overeating, reductions in energy-expending activities and a hypogonadal condition. The Prader-Labhart-Willi syndrome presents a similar set of symptoms including hyperphagia, hypogonadalism, hypotonia and obesity. The parents of Prader-Labhart-Willi children report that they seem insensitive to pain. Moreover, naloxone (an opioid antagonist) reduced the hyperphagia of 2 male patients with Prader-Labhart-Willi syndrome as reported by Kyriakides and colleagues[4]. These indications all seem compatible with the hypothesis of excessive opioid activity. Therefore we measured the quantity of ir-β-ep in the plasma of 6 patients with the Prader-Labhart-Willi syndrome and 7 of their normal siblings.

The subjects with the syndrome ranged in age between 12 and 28 years. Four of them were females and 2 were males. All the subjects were obese with a history of intense