is suggested that the complexing species is the 8-quinolinol molecule and not its anion. This conclusion is based on the fact that, in the pH 1.5–2.0 range, the anion concentration is negligible as computed from the acid dissociation constant of the 8-quinolinol in the specific solvent.

Variation of the solvent affected significantly the composition, stability, and *d*-orbital splitting of the metal ion in the formed complex. Due to steric factors, no dimethylformamide molecules appear to coordinate in the inner coordination sphere of metal ion; as a result, the 1:3 vanadyl–8-quinolinol complexes are predominant. In 96% ethanol and dioxane–water, one solvent molecule coordinates in the inner coordination sphere and 1:2 complexes are formed.

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Isolation and Characterization of the Cardiotonic Polypeptide Anthopleurin-A from the Sea Anemone *Anthopleura xanthogrammica*

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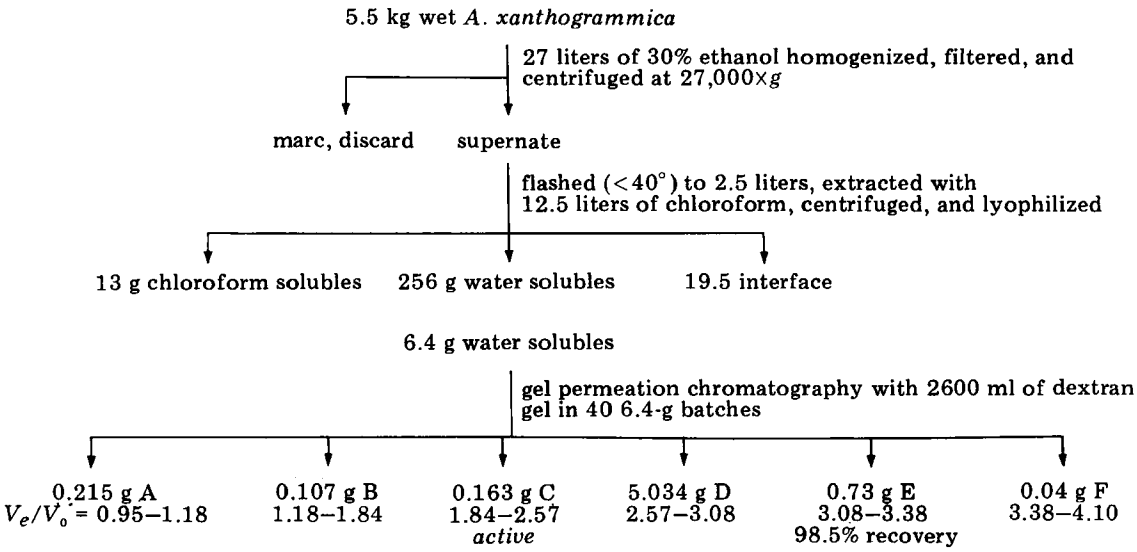
Abstract □ A highly potent cardiotonic polypeptide, anthopleurin-A, was isolated from the sea anemone, *Anthopleura xanthogrammica* (Brandt), using solvent partition, gel permeation chromatography, and cation-exchange chromatography. It is a pure basic polypeptide with a molecular weight of about 5500.

Keyphrases □ Anthopleurin-A—isolated from sea anemone *Anthopleura xanthogrammica* extract □ *Anthopleura xanthogrammica*—sea anemone extract, cardiotonic polypeptide anthopleurin-A isolated □ Cardiotonic polypeptide—anthopleurin-A isolated from sea anemone □ Sea anemone—extract, cardiotonic polypeptide anthopleurin-A isolated

Several extracts of coelenterates have been examined in this laboratory for antitumor activity (1–4) and, more recently, for a positive inotropic effect (4, 5). Interest was first stimulated in *Anthopleura xanthogrammica* when the crude extract was found to be highly toxic when administered intravenously to an anesthetized rat[1]. Later, the crude extract at much higher dilutions exhibited a strong positive inotropic effect on isolated rat atria; pretreatment with adrenergic receptor blocking agents, 6-hydroxydopamine or reserpine, did not affect the response, indicating that the extract acts by a nonadrenergic mechanism (5).

Many investigators have examined the chemical nature and pharmacology of toxins from coelenterates (6). An extract of the tentacles of *Calliactus polypus* (a sea anemone) caused a brief lowering of coronary outflow, heart rate, and amplitude of cardiac contractions in the isolated rabbit heart, and higher doses produced irregular cardiac contractions, usually resulting in cardiac

[1] Dr. George W. Read and Ms. Charlotte Oda, Department of Pharmacology, University of Hawaii, personal communication.



Scheme I—Extraction and initial purification scheme from A. xanthogrammica *to Fraction C*

arrest (7). It was reported[2] that extracts of about 15% of the species from five phyla of marine animals showed an increase in contractile force in the Langendorf guinea pig heart preparation and that a compound isolated from the polar fraction of the sponge, *Dasychalina cyathina,* is capable of reversible cessation of mechanical activity in the same preparation.

Low molecular weight polypeptides such as bradykinin and its congeners have been studied with respect to their cardioactivity (8). The question of whether these agents increase the heart rate and myocardial contractile force directly or indirectly through reflex is still moot (8). Recently, an inotropically active peptide from dog blood plasma was partially identified (9) as an "intact molecule" attached to an octapeptide (seven isoleucine and one glycine residues) and had activity parallel to that of epinephrine on the dog heart papillary muscle.

Although the cardiac glycosides have long been recognized as effective cardiotonic agents, they all have caused serious side effects such as arrhythmias and cardiac arrest. It, therefore, seemed advisable to pursue the isolation and pharmacological study of the cardiotonic substance(s) in *A. xanthogrammica.*

EXPERIMENTAL

Extraction—*A. xanthogrammica* (Brandt) specimens were collected from Bodega Bay, Calif.[3], preserved in 95% ethanol, and stored at 4° prior to extraction. Five different collections, made at different times of the year, contained a consistent amount of the cardiotonic substance.

Wet, drained anemones (5.5 kg) were cut into <2-cm pieces and homogenized in batches in a blender for 5 min with 27 liters of 30% ethanol; this volume included the ethanol used to preserve the specimens. The mixture was allowed to stand for 1 week at 20° with occasional stirring and was then filtered through six layers of cheesecloth. The filtrate was flash evaporated at ≤40° to about 2.5 liters and partitioned with 12.5 liters of chloroform, in batches, by thorough agitation followed by centrifugation at 27,000×*g* for 30 min. This procedure produced 13 g of chloroform solubles, 10 g of interface solids, and 256 g (lyophilized weight) of water solubles (contractile force in the isolated rat atria increased 240% in 20 min at 12 ppm in the atria bath; see Ref. 5 for details of the assay method).

Gel Permeation Chromatography—The crude extract was split into 40 equal portions for chromatographic separation on cross-linked dextran gel[4] (Fig. 1). A column, 53 × 8.3 cm, containing 2600 ml of wet gel (V_0 = 825 ml) was equilibrated with 0.1 *M* NH_4HCO_3 saturated with chloroform (to prevent microorganism growth). Fifty milliliters of water containing 6.4 g of crude extract was placed on the column and eluted with the 0.1 *M* NH_4HCO_3 at 8–9 ml/min at room temperature. Scheme I gives the weights and V_e/V_0 values for the lyophilized fraction. The bioassays for contractile force of the isolated rat atria, carried out as in Ref. 5, showed no activity in Fractions A, E, and F. The bioassays of Fractions B, C, and D were as follows:

fraction	concentration, ppm	increase in contractile force (time to maximum), %
B	4	0
	40	100 (6 min)
C	0.4	120 (5 min)
D	4	0
	40	30 (4 min)

Fraction C contained ~99% of the cardiotonic activity; 6.4 g of Fraction C was produced.

Ion-Exchange Chromatography—Fraction C, 610 mg, in 5 ml of 0.03 *M* phosphate buffer at pH 7.5, was put on a cation-exchange column[5], 48 × 4 cm. The column contained 600 ml of wet resin equilibrated with the same buffer saturated with chloroform (V_0 = 205 ml). A stirred reservoir of 1500 ml of the buffer was connected to the column. After 110 ml of buffer had flowed through the column, the reservoir volume was maintained constant by feeding with a 0.03 *M* phosphate buffer in 0.5 *M* NaCl adjusted to pH 7.5 for gradient elution. Both solutions were saturated with chloroform. The flow rate was about 3 ml/min, and the run was made at room temperature.

The effluent was monitored using UV absorbance at 280 nm[6]. Only two cuts contained activity: Compound I, V_e/V_0 2.99–3.39; and Compound II, V_e/V_0 6.30–7.01 (Fig. 2). The I cut (Fraction G) was immediately lyophilized (610 mg, including the salts), and the II cuts were frozen and accumulated for future work. Immediately following the II fraction, 25 ml of 10% NaCl was put on the column followed by the 0.03 *M* phosphate buffer (no sodium chloride) to clean the column and reequilibrate it for the next run. Fraction G was kept at −20° and was used for all pharmacological studies.

[2] S. G. Zelenski, A. J. Weinheimer, and P. N. Kaul, Fourth Food-Drugs from the Sea Conference, University of Puerto Rico, Mayaguez, Puerto Rico, Nov. 17–21, 1974.

[3] The authors thank Dr. Cadet Hand, Director, Bodega Marine Laboratory, University of California, Bodega Bay, Calif., for supplying the collections of the sea anemones.

[4] Sephadex G-50, Pharmacia Fine Chemicals.

[5] CM-Sephadex C-25, Pharmacia Fine Chemicals.

[6] Model UA-5 absorbance monitor, Instrument Specialties Co., Lincoln, NE 68505

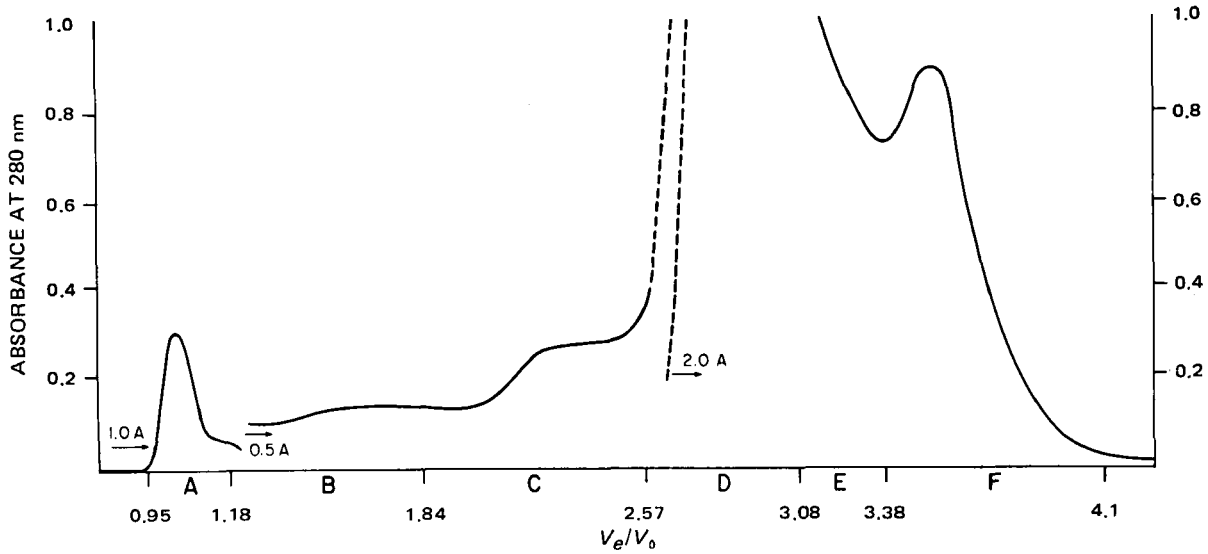


Figure 1—*Gel permeation chromatography of the crude extract of* A. xanthogrammica. *The notations 1.0A, 0.5A, and 2.0A indicate the points at which full-scale sensitivity on the chart was switched to these absorbance values.*

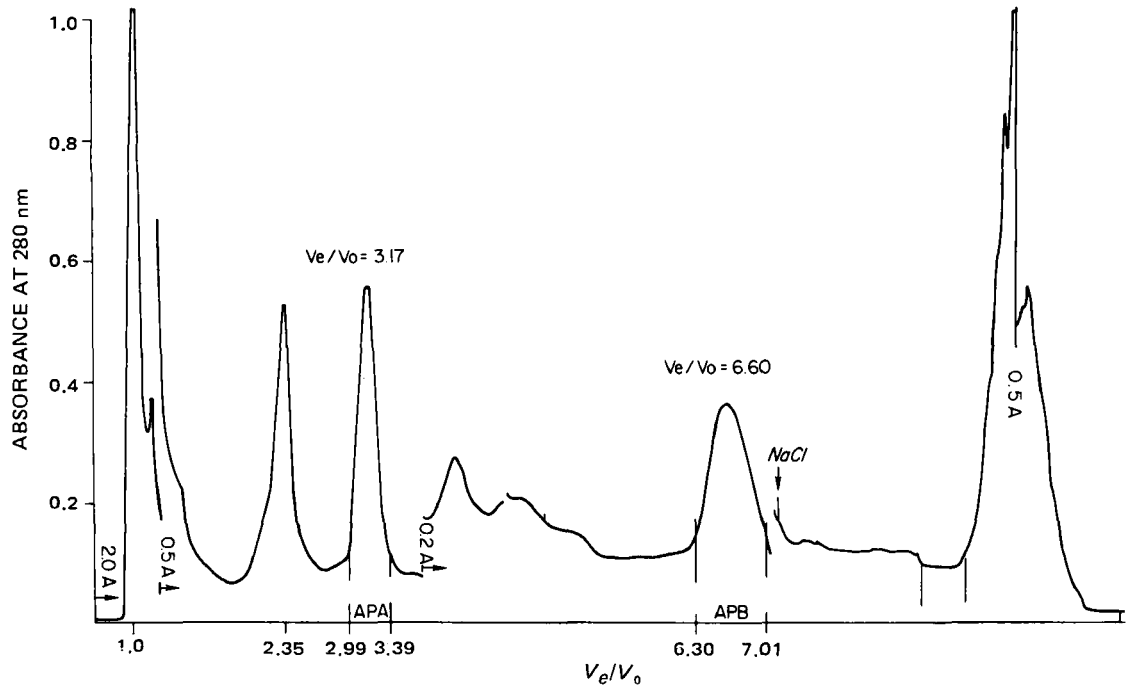


Figure 2—*Cation-exchange of Fraction C to give Fraction G [anthopleurin-A (APA)] and anthopleurin-B (APB). The notations 2.0A, 0.5A, and 0.2A indicate the points at which full-scale sensitivity on the chart was switched to these absorbance values.*

Compound I showed no signs of deterioration when kept at −20° for 1 year as lyophilized Fraction C or as lyophilized Fraction G. As Fraction G, it is stable for at least 24 hr at 20.5° in 10^{-7} *M* solutions at pH 4.55–7.80 but shows about 90% decomposition at pH above 11 (Table I).

Purification—A 50-ml buret (54 × 1.2 cm) containing 52 ml of wet dextran gel[4] was equilibrated with 0.017 *M* acetic acid in 0.03 *M* NaCl saturated with chloroform (V_0 = 21.1 ml). A solution of 102.6 mg of Fraction G was dissolved in 0.4 ml of water, put on the column, and eluted at room temperature with the equilibrating solution. Fraction H was collected at V_e/V_0 1.66–2.16. No UV-absorbing material appeared before Fraction H and only a trace appeared thereafter (Fig. 3). Fraction H was lyophilized and weighed 20 mg (including the sodium chloride).

The salt was removed using a dextran gel[7] column. If water alone was used for elution, only part of I eluted and in an erratic pattern. The 20 mg of Fraction H was dissolved in 0.3 ml of 10% NaCl and put on a 59 × 1.2-cm column of 54 ml of wet dextran gel[7] equilibrated with 0.017 *M* acetic acid saturated with chloroform. The sample was followed by 0.5 ml of 10% NaCl and then the equilibrating solution. Compound I emerged completely and sharply at void volume (V_e/V_0 0.93–1.12) and was salt free. It was immediately lyophilized to give 1.4 mg of pure anthopleurin-A (Fraction I).

For electrophoretic studies and amino acid analysis, the ion-exchange elution on both dextran gel columns was repeated (with about 60% overall recovery) starting with Fraction I. The column reruns were carried out in a fashion identical to the preparation of Fractions H and I to give the doubly purified Compound I (Fraction J). Figure 4 shows that the second scan[4] lacked extraneous material. Compound I as Fraction J did not crystallize from evaporation of a water solution in which it was very soluble, possibly due to a mixture of hydrates, but gave a colorless amorphous powder when an aqueous solution was lyophilized. If a molecular weight of 5500 is assumed, Fraction J has an ED_{50} for positive inotropic effect (isolated guinea pig atria) at 10^{-9} *M* in the atria bath (see Ref. 5 for method).

[7] Sephadex G-10, Pharmacia Fine Chemicals.

Table I—Effect of pH on Cardiotonic Activity of 10^{-7} *M* Anthopleurin-A

Sample	pH[a]	Incubation Period at 20.5°, hr	Concentration to Give Positive Inotropic Effect, *M*	
			Threshold	Maximum
1	4.55	2	5×10^{-10}	10^{-9}
2	4.55	24	5×10^{-10}	10^{-9}
3	6.78	2	5×10^{-10}	10^{-9}
4	6.78	24	5×10^{-10}	10^{-9}
5	7.8	2	10^{-9}	5×10^{-9}
6	7.8	24	10^{-9}	5×10^{-9}
7	11.15	2	10^{-9}	5×10^{-9}
8	11.15	24	5×10^{-9}	10^{-8}
9	11.15	2	5×10^{-9}	10^{-8}
10	11.75	24	5×10^{-9}	10^{-8}

[a] Using 0.1 *M* NaH_2PO_4 with incremental sodium hydroxide additions.

Electrophoretic Studies—For the acidic, basic, and sodium lauryl sulfate gel electrophoresis, commercial precast 12% acrylamide gels[8] and matched buffer systems were used.

Acid Disc Gel Electrophoresis—For the gel labeled AP-A + DTE, the sample was prepared by mixing 5 μl (containing 25 μg) of Compound I aqueous solution with 5 μl of 0.5 *M* dithioerythritol (adjusted to pH 8.7 with ethylenediamine) and allowing the mixture to stand for 2 hr. The mixture was then acidified with 2 μl of 1% acetic acid, and 3 μl of the tracking dye (0.5% methyl green in 30% sucrose, 0.4 *M* glycine–acetic acid at pH 3.6) was added. The total volume was 15 μl.

For the gel labeled AP-A + NH_3, 10 μl (containing 25 μg) of Compound I aqueous solution was mixed with 10 μl of 1 *M* NH_3 and heated at 60° for 1 hr. The sample was evaporated to dryness (nitrogen), and 10 μl of 0.1% acetic acid and 5 μl of the methyl green tracking dye were added. Ten microliters of the mixture was used.

The prepared samples were each carefully layered on top of the buffered gel under the buffer (0.188 *M* in both glycine and acetic acid, pH 3.5) in an electrophoresis bath[9] with 1500 ml of buffer in the bottom cathode compartment and 500 ml of buffer in the top anode compartment. The power was turned on without delay and set at 100 v (1 mamp/tube) until the samples had entered the gel completely (about 5 min). The voltage was then increased to 200 v (2 mamp/tube) until the tracking dye was within about 5 mm of the cathode end (bottom) of the tube (about 90 min).

The gel tubes were quickly pulverized using a "gel tube eliminator"[8], the end of the gel was cut off directly through the center of the dye, and each gel was placed in 50 ml of a 10% acetic acid–40% 2-propanol–50% water solution and gently swirled for 5 hr to fix the polypeptide. The shrunken gels were then placed in 1.5 × 10-cm test tubes with 22 ml of 0.25% anazolene sodium[10] in 7% acetic acid for 12 hr. The gels were transferred to the destainer[9] and washed with 7% acetic acid for 72 hr. The results are shown in Fig. 5.

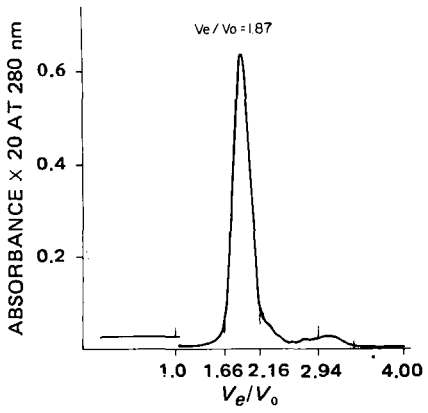


Figure 3—*Gel permeation chromatography of Fraction G to give Fraction H.*

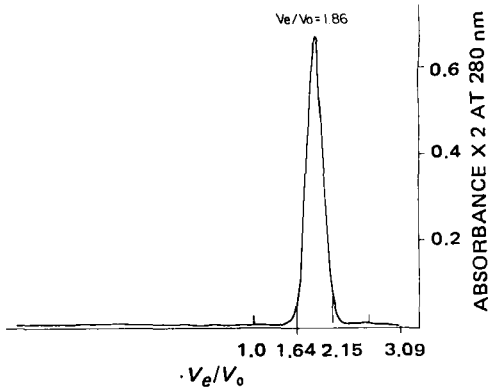


Figure 4—*Gel permeation chromatography of repurified Fraction H.*

Basic Disc Gel Electrophoresis—For basic gels, the procedure was the same as already described except that the anode was the bottom compartment, the buffer was pH 8.9 (0.188 *M* in both glycine and tromethamine), and the tracking dye was 0.5% bromphenol blue in 30% sucrose (0.05 *M* in both tromethamine and glycine[8]). A sample of 15 μl (containing 15 μg) of Compound I aqueous solution plus 3 μl of tracking dye was placed on the equilibrated gel and run as with the acid gel. The gel showed no bands.

Sodium Lauryl Sulfate Electrophoresis—For sodium lauryl sulfate-treated polypeptides and proteins, the procedure was as described previously[8]. The buffer was 0.205 *M* in both tromethamine and acetic acid and contained 0.1% (w/v) sodium lauryl sulfate, pH 6.4 in buffer and 6.1 in the gel. The buffer was introduced into the prepared gel at 100 v (constant voltage) and required about 6 hr. The buffer in the cathode compartment (top) was then replaced with fresh buffer. The samples were then introduced at 60 v (4 mamp/tube) and run at 105 v (7.5 mamp/tube). The incubating sodium lauryl sulfate solution was 0.04 *M* in both tromethamine and acetic acid, 0.04 *M* in dithioerythritol, and 0.001 *M* in edetic acid and contained 1% (w/v) sodium lauryl sulfate.

The samples for all three gels shown in Fig. 6 were prepared by incubating 10 μl of the protein or polypeptide solution with 10 μl of the incubating sodium lauryl sulfate solution at 65° for 30 min. Then 4 μl of the bromphenol blue tracking dye was added, and the run was carried out as already described. Fixing, staining, and destaining were performed as with the acid gel. The Compound I solution concen-

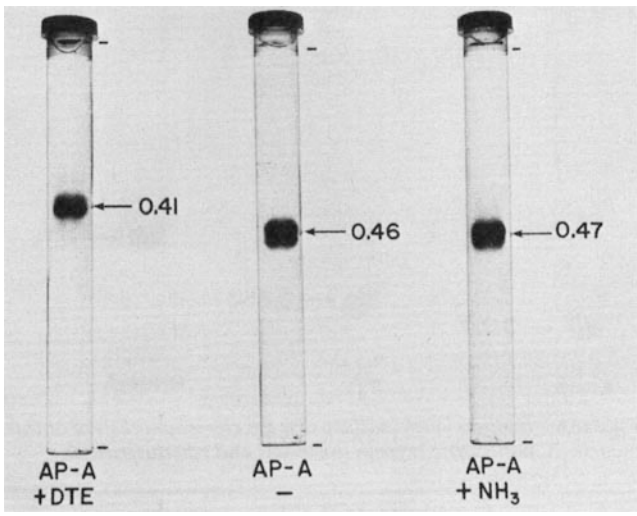


Figure 5—*Disc gel electrophoresis at pH 3.6. (Bottom was the cathode.)*

[8] Bio-phore precast gels, matched buffer systems, and other supplies from Bio-Rad Laboratories, Richmond, Calif. The buffers were introduced as described in their Bulletin 1024, with SDS addendum, Apr. 1974.

[9] Hoefer Scientific Instruments, San Francisco, CA 94107

[10] Coomassie Brilliant Blue R-250, Bio-Rad Laboratories, Richmond, Calif.

Table II—Amino Acid Analysis of Anthopleurin-A[a]

Amino Acid	Micromoles × 10			Average[b] or Extrapolation[c]	Micromoles[a] ÷ 0.0227	Closest Integer
	At 8 hr	At 12 hr	At 16 hr			
Aspartic acid	1.07	1.07	1.07	1.07[b]	4.71	5
Threonine	0.67	0.70	0.70	0.69[b]	3.04	3
Serine	1.17	1.12	1.10	1.18[c]	5.19	5
Glutamic acid	0.73	0.73	0.74	0.73[b]	3.21	3
Proline	1.12	1.12	1.11	1.12[b]	4.93	5
Glycine	1.89	1.88	1.87	1.88[b]	8.27	8
Alanine	0.44	0.44	0.44	0.44[b]	1.94	2
Cystine/2	1.10	1.11	1.03	1.35[c]	5.94	6
Valine	0.49	0.48	0.48	0.48[b]	2.11	2
Methionine	0.03	0.01	0.01	0.03[d]	0.13	0
Isoleucine	0.23	0.23	0.24	0.23[b]	1.01	1
Leucine	0.83	0.83	0.82	0.83[b]	3.65	4
Tyrosine	0.35	0.33	0.30	0.42[c]	1.85	2
Phenylalanine	0.19	0.21	0.19	0.20[b]	0.88	1
Lysine	0.51	0.49	0.50	0.50[b]	2.20	2
Histidine	0.34	0.35	0.35	0.35[b]	1.54	2
Ammonia	1.42	—[e]	1.58	1.26[f]	5.54	6
Arginine	—[e]	0.40	0.40	0.40[b]	1.76	2
Tryptophan	0.05	(24-hr barium hydroxide hydrolysis)		0.05	0.22	0

[a] Anthopleurin-A, 125 μg (0.0227 μmole), was used. [b] Average of the three values. [c] Extrapolated from 12- and 16-hr values. [d] Eight-hour value. [e] Spurious result. [f] Extrapolated from 8- and 16-hr values.

tration was 1 μg/μl (10 μg total), the pancreatic trypsin inhibitor[11] concentration was 2 μg/μl (20 μg total), and the ribonuclease A[11] concentration was 2 μg/μl (20 μg total), all in water.

When I was run by adding the sodium lauryl sulfate incubating solution and immediately putting it on the gel (0 incubation time), an intense single band at R_f 0.86 was seen. With 1 hr of incubation, the gel appeared the same as shown in Fig. 6 with a trace at R_f 0.86 and the dense band at 0.91.

Electrofocusing—To determine the isoelectric point and as a check of purity by electrofocusing, a modification of the procedure described by Wrigley (10) was employed. The 10% gels were prepared in 6.0 (i.d.) × 77-mm silica tubes using the following solutions: 1.16 ml of water, 0.67 ml of 30% acrylamide[12], 0.033 ml of ampholytic surfactant[13], 0.022 ml of ampholytic surfactant[14], and 0.11 ml of potassium persulfate (1% $K_2S_2O_8$ in water).

All solutions except the surfactants were deaerated by vacuum boiling and handled under nitrogen. The potassium persulfate was added last, and the well-mixed solution was placed in the tubes. About 200 μl of deaerated water was immediately layered on top of the solution. Polymerization was complete in 10–15 min, and gels were used within 30 min. The presence of oxygen may completely inhibit the polymerization.

The 5% sucrose solution for the top of the gel consisted of 0.10 ml of 40% ampholytic surfactant[13], 0.50 ml of 40% sucrose, and 3.40 ml of water.

The anode compartment (top) contained 500 ml of 0.2% H_2SO_4, and the cathode compartment (bottom) contained 1500 ml of 0.4% ethylene diamine.

The bottom compartment was filled, the gels were put in place, and the 5% sucrose solution was put on top of the gel. The sample containing 50 μg of I in 10 μl of 0.1% acetic acid was mixed with 3.3 μl of 40% sucrose and layered under the 5% sucrose and onto the gel. The power was immediately turned on at 75 v (2.5 mamp/tube) and allowed to rise to 400 v, where it was kept at constant voltage until the end of the run (1.0 mamp/tube at the end of the run) (85 min). The UV detector[6] was coupled with a gel scanner[15], and the gel was immediately scanned (solid line in Fig. 7).

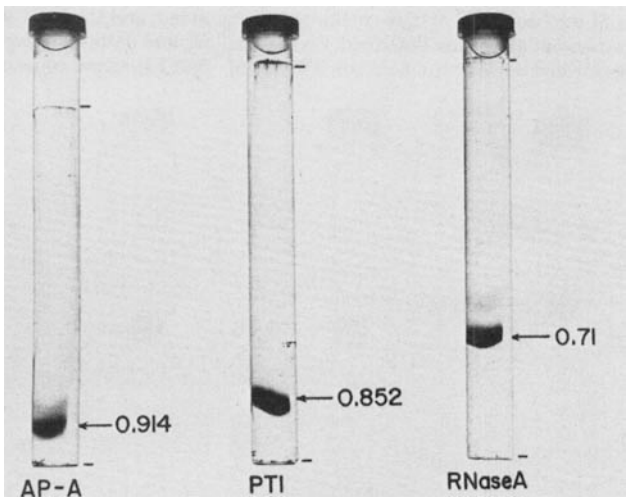


Figure 6—*Sodium lauryl sulfate disc gel electrophoresis of anthopleurin-A, pancreatic trypsin inhibitor, and ribonuclease A.*

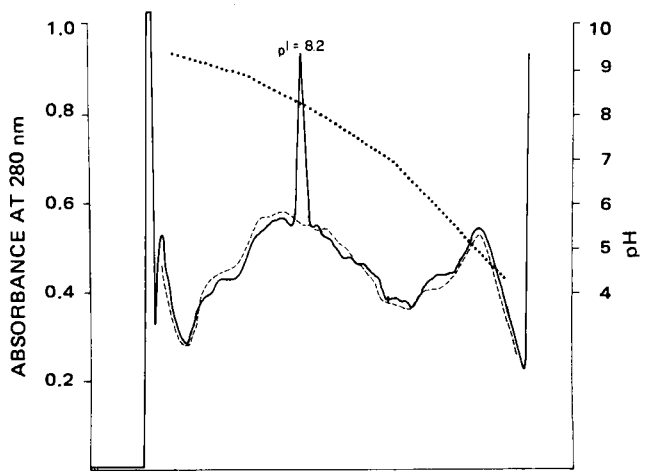


Figure 7—*Gel scan of gel isoelectric focusing of anthopleurin-A* (—), *blank ampholytes* (- - - -), *and pH of the gel* (. . . .).

[11] The authors are indebted to Dr. J. F. Lenney of the Pharmacology Department, University of Hawaii, for the pancreatic trypsin inhibitor and ribonuclease A (RNase A) samples.

[12] Nine grams of acrylamide, 0.03 g of *N,N'*-methylenebis(acrylamide) in 30 ml of solution.

[13] Ampholine carrier 3-10 ampholytes, 40%, pH 3-10, LKB Produkter AB, S 161 25 Bromma 1 Sweden.

[14] Bio-Rad 9-10 ampholytes, 40%, pH 9–10, Bio-Rad Laboratories, Richmond, Calif.

[15] Isco model 659 gel scanner, Instrument Specialties Co., Lincoln, NE 68505

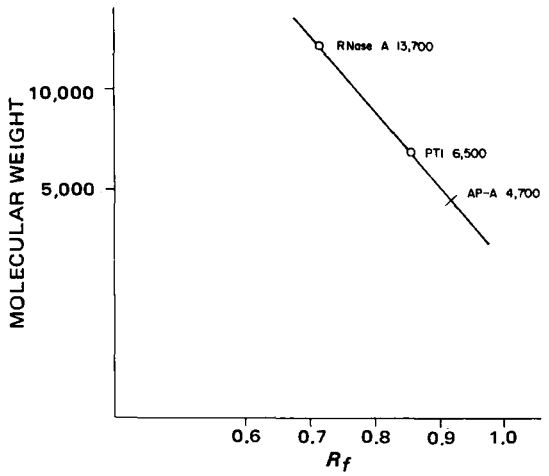


Figure 8—*Semilog plot of* R_f *values from Fig. 5* versus *molecular weight.*

A second gel was prepared similarly, except that no I was put on it. This gel was chilled (0°) and then scanned immediately after the I gel as a control (dashed line in Fig. 7). The gel with the I samples was then sliced into 10 equal length cylinders. Each slice was placed in 1.5 ml of water and allowed to stand for 2 hr. The pH of each tube was then measured: first (anode end), 4.35; second, 5.1; third, 6.0; fourth, 6.83; fifth, 7.35; sixth, 8.02; seventh, 8.40; eighth, 8.85, ninth, 9.13; and 10th (cathode end), 9.35. These results were plotted directly on the UV absorbance monitor readout (dotted line in Fig. 7) at 5, 15, 25, *etc.* to 95% of the duration of the gel scan (abscissa). Compound I gave a sharp peak at pH 8.2 (Fig. 7).

Amino Acid Analyses—A 1.0-mg sample of Compound I (Fraction J) was dissolved in 200 μl of water. Fifty-microliter samples of this solution were quantitatively transferred to four 3-ml ampuls. One milliliter of redistilled 6 *N* HCl was put in three of these ampuls, and 1 ml of 0.2 *M* $Ba(OH)_2$ was placed in the fourth (for tryptophan analysis). All of the ampuls were fire sealed under vacuum.

The three acid samples were placed in an oven at 112 ± 1.0°. One sample at a time was removed after 8, 12, and 16 hr; they were opened and placed over sodium hydroxide pellets in a vacuum. It had previously been determined that hydrolysis was almost complete at 6 hr. The barium hydroxide sample was heated at 128 ± 1.0° for 24 hr for the tryptophan analysis. The contents were then neutralized to pH 6.5 with 0.2 *M* H_2SO_4, centrifuged to remove barium sulfate, and then placed in a vacuum over sodium hydroxide pellets. The samples were dry in 48 hr.

Amino acid analyses[16] were carried out on an automatic amino acid analyzer[17] with acid hydrolysates split for first the acid and neutral amino acids and then the basic amino acid analyses (Table II). Standards containing 0.100 μmole of each amino acid were run just prior to the Compound I hydrolysates.

RESULTS AND DISCUSSION

Gel filtration of the crude extract from *A. xanthogrammica* gave a 40-fold increase in purity in the V_e/V_0 1.84–2.57 Fraction C (Scheme I). Cation exchange, using phosphate buffer at pH 7.5 and gradient elution with 0.5 *N* NaCl, provided an excellent separation of anthopleurin-A (I) in a purity of about 95% (based on UV absorbance areas of impurities seen in preparation of analytically pure material) (Fig. 2). When Fraction G was lyophilized, it contained 1–1.4 mg of polypeptide/100 mg total weight, the balance being sodium chloride and phosphate salts. This material was used for all pharmacological studies, details of which are reported elsewhere (11).

As shown in Fig. 2, there was a second active substance, anthopleurin-B (II) at V_e/V_0 6.30–7.01. This substance also showed a strong positive inotropic effect and no chronotropic effect at concentrations equivalent to I based on UV absorbance, assuming equal molar absorptivities. The characteristics of II have yet to be examined.

All trial separation procedures were monitored by bioassay, using the isolated rat atria for the determination of positive inotropic effects (5). Over 700 such bioassays were carried out during the development of this procedure. About 90% of the activity in the crude extract was recovered in Fraction G.

Qualitative tests of Fraction I indicated the presence of the polypeptide based on ninhydrin-positive spots, using paper chromatography of a hydrolysate, and the absence of sugars by the α-naphthol–sulfuric acid method.

Isoelectric focusing of Fraction J indicated a pI = 8.2 and the absence of any UV-absorbing impurities (Fig. 7). Disk gel electrophoresis at pH 3.6 gave an R_f value of 0.46 based on methyl green as the tracking dye and indicated lack of anazolene sodium dyeable impurities (Fig. 5). Compound I would not enter a basic gel at pH 8.9, indicating a possible lack of free carboxyl groups. By using sodium lauryl sulfate and incubation with dithioerythritol, gel electrophoresis indicated a molecular weight of about 4700 based on R_f values determined for ribonuclease A and pancreatic trypsin inhibitor. With the same solution, but zero incubation time, the R_f value corresponded to a molecular weight of about 6500, indicating the possible loss of a portion of the molecule by disulfide linkage splitting. No dyeable impurities were seen in either case.

Molecular weight determinations of polypeptides with molecular weights below 10,000–15,000 using the sodium lauryl sulfate technique are not considered reliable (12). However, the molecular weights based on the amino acid analysis (5500) and the zero incubation time sodium lauryl sulfate technique (6500) were not grossly disparate. Incubation with dithioerythritol changed the pH 3.6 gel electrophoresis R_f value from 0.46 to 0.41, again indicating that a segment of the molecule was lost and that the remaining residue was less basic than the original I; incubation with 1 *M* NH_3 did not change the R_f value (Fig. 8). Splitting of I by dithioerythritol incubation alone at pH 8.7 completely destroyed the activity.

The amino acid analyses showed that there were traces of methionine and tryptophan, apparently from impurities. However the other amino acids were recovered in good yields and with a reasonable integer fit. Histidine was a notable exception, with persistent 1.5-mole values. The moles of amino acids were based on the actual weight of I (125 μg) and a molecular weight of 5500, rather than the closest integer fit. Compound I was not retarded on G-10[7] (exclusion limit 700), was slightly retarded (peak $V_e/V_0 = 1.14$) on G-25[18] (exclusion limit 5000), and was markedly retarded (peak $V_e/V_0 = 1.87$) on G-50[4] (exclusion limit 30,000); also the molecular weight from incubated sodium lauryl sulfate electrophoresis was about 4700. Because of these data, the value of 5500 rather than 11,000 was chosen as the probable molecular weight. Compound I has 53 amino acids, with an excess of four basic groups over the number of acidic groups (Table II). The amino acid analysis can only be considered tentative until sequencing is done[19].

At this time it appears that I is a basic polypeptide containing 53 amino acids, with two segments (both inactive) separable by disulfide (cystine) splitting, and possibly containing no free carboxyl groups. It is relatively stable at neutral and lower pH values and is very soluble in deionized water.

The pharmacological data, appearing elsewhere in detail (11), indicate that I is about 200–1000 times as potent as ouabain in positive inotropic effect, having an ED_{50} at 10^{-9} *M* on isolated guinea pig atria. The LD_{50} in mice (intraperitoneal) is about 0.3–0.4 mg/kg. It does not show any chronotropic effect when pure (as Fraction G). It will reverse completely ouabain-induced cardiac arrest. At 5×10^{-7} *M* concentrations of Compound I, none of the following enzymes is inhibited: sodium or potassium adenosine triphosphatase, monoamine oxidase, catechol *O*-methyltransferase, and cyclic 3′,5′-nucleotide phosphodiesterase; nor does I affect the 3′,5′-cyclic adenosine monophosphate content of the guinea pig heart. Compound I is not nearly as sensitive to the Ca^{+2} concentration as is ouabain, and lowered external K^+ does not alter drug toxicity as with ouabain. Compound I has been shown

[16] The authors thank Dr. Suresh S. Patil, Plant Pathology Department, University of Hawaii, for the use of the instrument and Mr. Phillip D. Youngblood for running the analyses.

[17] Beckman model 116 amino acid analyzer, Beckman Instruments, Spinco Division, Palo Alto, CA 94304

[18] Sephadex G-25, Pharmacia Fine Chemicals.

[19] *Note added in proof:* The sequencing of I has been completed by M. Tanaka *et al.* (Department of Biochemistry, University of Hawaii), and a manuscript is in preparation. The sequence indicates the following amino acid composition: ASP_4 THR_3 SER_6 GLU PRO_4 GLY_8 ALA CYS_6 VAL_2 ILE LEU_4 TYR LYS_2 HIS_2 ARG TRP_3 $(NH_3)_3$.

to be effective in the isolated heart in rats, guinea pigs, rabbits, cats, and dogs and in the *in situ* heart in dogs and cats.

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Hypocholesterolemic Agents V: Inhibition of β-Hydroxy-β-methylglutaryl Coenzyme A Reductase by Substituted 4-Biphenylylalkyl Carboxylic Acids and Methyl Esters

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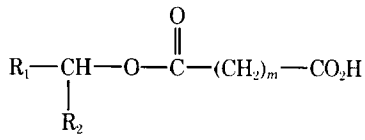
Abstract □ Eleven substituted 4-biphenylylalkyl carboxylic acids and three methyl esters were synthesized and assayed for inhibition of rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase. Five of the acids were analogs, resulting from various isosteric replacements of the carbonyl and ether oxygens of the previously described reversible inhibitor 1-(4-biphenylyl)pentyl hydrogen succinate. No significant change in activity was noted, except upon introduction of an amide linkage where a decrease in inhibition was found. Six carboxylic acids and three methyl esters, all containing the 4-biphenylyl radical but lacking the *n*-butyl side chain found in 1-(4-biphenylyl)pentyl hydrogen succinate, also were inhibitors of the reductase.

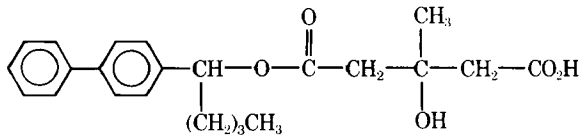
Keyphrases □ β-Hydroxy-β-methylglutaryl coenzyme A reductase—effect of 4-biphenylylalkyl carboxylic acids, rat liver microsomes □ 4-Biphenylylalkyl carboxylic acids—synthesis, effect on β-hydroxy-β-methylglutaryl coenzyme A reductase, rat liver microsomes □ Enzymes—β-hydroxy-β-methylglutaryl coenzyme A reductase, effect of 4-biphenylylalkyl carboxylic acids, rat liver microsomes □ Hypocholesterolemic agents, potential—4-biphenylylalkyl carboxylic acids synthesized, effect on β-hydroxy-β-methylglutaryl coenzyme A reductase, rat liver microsomes □ Structure–activity relationships—4-biphenylylalkyl carboxylic acids synthesized, effect on β-hydroxy-β-methylglutaryl coenzyme A reductase

In previous reports (1, 2), an approach to the design of inhibitors of cholesterol biosynthesis as potential hypocholesterolemic agents was discussed. The rationale for the inhibition of the enzyme, β-hydroxy-β-methylglutaryl coenzyme A reductase, was presented (1). These studies led to the discovery that maximum inhibition was obtained in a series of arylalkyl hydrogen alkanedioates (I), where R_1 = biphenyl, R_2 = *n*-butyl, and m = 1–4.



I

Modification of the acid portion of the glutarate analog of I by the incorporation of a β-hydroxy-β-methyl moiety provided II. Analog II, which closely resembles the acid portion of the substrate, β-hydroxy-β-methylglutaryl coenzyme A, was seven times more active than the glutarate analog of I and is the most active inhibitor found thus far.



II

This paper describes the synthesis and assay of compounds where isosteric replacements of the various atoms of the ester function of I were made. In addition, reports by Eades *et al.* (3, 4) indicated that a series of 4-substituted biphenyl derivatives inhibited the incorporation of 1-^{14}C-acetate into cholesterol in the *in vitro* rat liver homogenate system and decreased serum